AG-221, a First-in-Class Therapy Targeting Acute Myeloid Leukemia Harboring Oncogenic IDH2 Mutations
ABSTRACT

Somatic gain-of-function mutations in isocitrate dehydrogenases (IDH) 1 and 2 are found in multiple hematologic and solid tumors, leading to accumulation of the oncometabolite (R)-2-hydroxylutarate (2HG). 2HG competitively inhibits α-ketoglutarate–dependent dioxygenases, including histone demethylases and methylcytosine dioxygenases of the TET family, causing epigenetic dysregulation and a block in cellular differentiation. In vitro studies have provided proof of concept for mutant IDH inhibition as a therapeutic approach. We report the discovery and characterization of AG-221, an orally available, selective, potent inhibitor of the mutant IDH2 enzyme. AG-221 suppressed 2HG production and induced cellular differentiation in primary human IDH2 mutation–positive acute myeloid leukemia (AML) cells ex vivo and in xenograft mouse models. AG-221 also provided a statistically significant survival benefit in an aggressive IDH2R140Q-mutant AML xenograft mouse model. These findings supported initiation of the ongoing clinical trials of AG-221 in patients with IDH2 mutation–positive advanced hematologic malignancies.

SIGNIFICANCE: Mutations in IDH1/2 are identified in approximately 20% of patients with AML and contribute to leukemia via a block in hematopoietic cell differentiation. We have shown that the targeted inhibitor AG-221 suppresses the mutant IDH2 enzyme in multiple preclinical models and induces differentiation of malignant blasts, supporting its clinical development. Cancer Discov; 7(5): 1–16. © 2017 AACR.

See related article by Shih et al., p. xxx.
See related commentary by Thomas and Majeti, p. xxx.

INTRODUCTION

Metabolic reprogramming is a hallmark of cancer, contributing to the initiation and maintenance of tumors (1, 2). The NADP⁺-dependent isocitrate dehydrogenases (IDH) are critical metabolic enzymes that interconvert isocitrate and α-ketoglutarate (αKG). Recurrent somatic point mutations in active site arginine residues of IDH1 (R132) and IDH2 (R140 and R172) have been found in multiple tumors, including acute myeloid leukemia (AML; refs. 3–9). Cancer-associated IDH1/2 mutations confer the neomorphic activity of reducing αKG to the oncometabolite (R)-2-hydroxylutarate (2HG; refs. 9–11). 2HG accumulation competitively inhibits αKG-dependent dioxygenases, including histone demethylases and methylcytosine dioxygenases of the TET family that regulate cellular epigenetic status (12–15). This epigenetic dysregulation is associated with impairment of cellular differentiation in multiple cell types, including hematopoietic cells (15–21). AGI-6780, a selective sulfonamide inhibitor of the mutant IDH2 enzyme, lowered 2HG levels and induced differentiation of TF-1 erythroleukemia cells and primary human AML cells harboring the IDH2R140Q mutation (17), providing in vitro evidence that inhibition of the mutant IDH2 enzyme can reverse some of the phenotypic changes it induces. Others have reported similar findings with mutant IDH1 inhibitor tool compounds in AML models (22).

IDH mutations are also found in premalignant disorders, including myelodysplastic syndromes (MDS), and were shown to drive leukemic transformation in cooperation with other genetic events in IDH-mutant mouse models of AML (23–25). It is possible that IDH-mutant cells drive clonal hematopoiesis, sustaining a reservoir of stem cells associated with resistance to conventional chemotherapy that needs to be targeted via alternative mechanisms (26–28).

Here, we report the design and characterization of AG-221 (enasidenib), an orally available, selective, potent, triazine inhibitor of the mutant IDH2 enzyme that dramatically reduced 2HG levels in multiple models. AG-221 induced differentiation in IDH2-mutant TF-1 cells and primary human AML cells ex vivo, as well as in four IDH2R140Q-mutant human AML xenograft mouse models in vivo, and provided a dose-dependent, statistically significant survival benefit in vivo in an aggressive human AML xenograft model, supporting its clinical development.
RESULTS

Discovery of Allosteric Inhibitors of the IDH2-Mutant Enzyme

In pursuit of drug candidates targeting recurrent oncogenic IDH2 mutations, we initiated a high-throughput screen for inhibitors of the enzyme carrying the most prevalent IDH2 mutation in AML, IDH2R140Q (29–31). Several triazine compounds active against the IDH2R140Q homodimer emerged, and initial hit-to-lead chemistry led to compound 1, the first sub–100 nmol/L inhibitor of IDH2R140Q (Fig. 1A). Although fairly potent in enzymatic and cellular assays, it displayed high lipophilicity, leading to solubility-limited absorption in vivo. In addition, its poor in vitro liver microsomal stability translated to high clearance in vivo. X-ray crystallography revealed binding of a compound 1 molecule to an allosteric site located within the homodimer interface of the IDH2R140Q-mutant enzyme, to which the selective sulfonamide inhibitor AGI-6780 also binds (17). These insights guided optimization of the substituents around the triazine core. Through the addition of mildly polar substituents, such as trifluoromethyl pyridine and 2-methyl-2-propanol, AG-221 (Fig. 1A and Supplementary Fig. S1A) was identified to have excellent potency for 2HG inhibition (Table 1), improved solubility, low clearance (0.83 l/h/kg), and good oral bioavailability (41%) in vivo in rats (Supplementary Fig. S1B and Supplementary Table S1).

AG-221 is a Slow Tight Binder of the IDH2R140Q-Mutant Enzyme

Because IDH2 is homodimeric, and somatic IDH mutations found in tumors occur in a single allele, with one wild-type (WT) allele, the mutant enzyme likely exists in cells as a mixture of mutant–WT heterodimers and mutant homodimers. As the heterodimer produces 2HG more efficiently than mutant homodimers (32), it is an important molecular target. We therefore characterized the activity of AG-221 against the heterodimer and mutant and WT homodimers.

AG-221 displayed a long residence time on the IDH2R140Q homodimer, with kinetics consistent with slow-onset tight binding inhibition (Supplementary Fig. S2A and S2B). AG-221 showed noncompetitive inhibition against the IDH2R140Q homodimer for the αKG substrate and uncompetitive inhibition against the IDH2R140Q and IDH2WT homodimers for NADPH and NADP⁺ cofactors, respectively (Supplementary Fig. S3A–S3C). AG-221 displayed time-dependent, nanomolar potency for inhibiting 2HG production by the IDH2R140Q homodimer (IC₅₀ = 0.10 μmol/L at 16 hours), the IDH2R140Q/WT heterodimer (IC₅₀ = 0.03 μmol/L), and the IDH2R172K/WT heterodimer (IC₅₀ = 0.01 μmol/L), and time-dependent, single-digit micromolar potency for inhibition of the canonical forward (oxidative) reaction in the IDH2WT homodimer (IC₅₀ = 1.8 μmol/L at 16 hours; Table 1). Similar potency was observed in the forward direction for the IDH2WT/R140Q and IDH2WT/R172K heterodimers, albeit with lower maximum percentage inhibition (range, 75%–64%). AG-221 displayed selectivity for IDH2-mutant homo- and heterodimers over IDH1WT- and IDH1R132H-mutant enzymes (Table 1), a panel of kinases (Supplementary Table 2; ref. 17), and a second panel of 80 receptors, ion channels, and enzymes (data not shown). Furthermore, AG-221 displayed excellent potency in reducing 2HG in cell lines ectopically expressing or overexpressing IDH2R140Q or IDH2R172K (Table 2). In these assays, AG-221 displayed higher potency against R140Q versus R172K, this was not observed in the enzyme assays.

AG-221 Stabilizes the Inhibitory Open Homodimer Conformation of IDH2R140Q

A high-resolution (1.55 Å) X-ray crystal structure of AG-221 in complex with IDH2R140Q, NADPH, and Ca²⁺ (IDH2R140Q-AG-221) confirmed that it binds to the allosteric site enclosed within the homodimer interface, and consequently the mutant enzyme adopts an open conformation. We observed alternative conformations for AG-221 binding in the pocket, owing to the pseudo 2-fold symmetric nature of the pocket (Fig. 1B). To understand the molecular mechanism of inhibition, we crystallized the 1.54 Å resolution X-ray structure of IDH2R140Q bound to substrate αKG, NADPH, and Ca²⁺ (IDH2R140Q-αKG). This catalytically primed complex adopts a compact closed homodimer conformation (Fig. 1C). Comparing the quaternary complexes of IDH2R140Q-AG-221 and IDH2R140Q-αKG suggested that AG-221 allosterically stabilizes the open homodimer conformation, preventing the conformational change required for catalysis, consistent with the mode of inhibition documented for IDH1R132H mutants (17, 33).

AG-221 binding is anchored by multiple hydrogen bonds and hydrophobic interactions within the pocket. The pocket is encapsulated by four helices (α9, α10, α9′, α10′) lining the sides, two loops (L1 and L1′), and the Y311–D312 interaction pairs capping the ends (Fig. 1D). Nitrogens at the 1, 3 positions on the diaminotriazine core accept hydrogen bonds from the amino sidechain of the Q316 residues, whereas linker amides donate hydrogen bonds to the Q316 carbonyl sidechain (Fig. 1B). The Q316 carbonyl also accepts a hydrogen bond from the 2-methyl-2-propanol moiety of AG-221. Other polar interactions include a halogen bond between AG-221’s trifluoromethylpyridine and the D312 capped residue. In addition, van der Waals interactions from surrounding hydrophobic residues W164, V294, V297, L298, V315, I319, and L320 contribute to AG-221’s high inhibitory potency. The dominant hydrophobic nature of the pocket reveals why larger polar substituents were less favorable, owing to a steric clash or desolvation energetic penalty. Along with the domain motions, movement of L1 and L1’ would be required to provide access to the binding site (17). The collective structural rearrangements needed to access the deeply buried pocket, combined with the multitude of interactions upon binding, explain the slow-on/slow-off tight binding kinetics of AG-221.

AG-221 Inhibited 2HG Production and Induced Differentiation in IDH2-Mutant TF-1 Cells and Primary Human AML Blasts

As reported previously (17), and consistent with observations in myeloblasts from patients with IDH1-2/mutant AML (34), IDH2R140Q expression in the TF-1 erythroleukemia cell line induced intracellular 2HG production to concentrations of 3,500 to 5,116 ng/10⁶ cells; intracellular and
Figure 1. AG-221 structure and binding characteristics. A, Key molecules leading to candidate AG-221 and corresponding in vitro and biochemical data. B, Co-complex crystal structure of IDH2R140Q homodimer with AG-221 bound to an allosteric site. Protein represented as ribbon (protomers colored cyan and green), AG-221 as magenta spheres, and NADPH as yellow sticks. C, Allosteric binding of AG-221 stabilizes inhibitory open conformation of the IDH2R140Q active site (left) versus the catalytically primed αKG-bound IDH2R140Q structure (right). Solvent-accessible surface for each protomer shown as translucent white area with cyan or yellow ribbon and solid green or yellow surface for IDH2R140Q:AG-221 and IDH2R140Q:αKG, respectively. αKG and NADPH shown in ball and stick representation, and AG-221 as solid spheres. D, Detailed view of AG-221 binding site at the IDH2R140Q dimer interface. Secondary structures flanking the compound shown as cylinders α9 and α9’ (residues 292–299), and α10 and α10’ (residues 310–325). Residues from each protomer shown in cyan/green. AG-221, shown as sticks (carbon in magenta, nitrogen in blue, fluorines in cyan), exhibits two possible asymmetric binding conformations. Residues Y311 and D312 (stick figures) cap one end of the pocket, and flexible loops L1 and L1’ (residues 151–168; ribbons) cap the other end. E, Molecular interactions of AG-221 in its binding site. Amino acid residues within a 3.5 Å radius of AG-221 shown as sticks (green/cyan for carbon atoms from adjacent homodimers). Hydrogen bond interactions between AG-221 and Q316 residue shown as dotted lines. Residues Q316, L320, I319, and V294 exhibit asymmetric alternative conformation in the binding site. Eh, hepatic extraction ratio; HLM, human liver microsome; inh, inhibitory.
extracellular 2HG levels were reduced by AG-221 treatment (Supplementary Fig. S4A and S4B). AG-221 also inhibited growth factor–independent proliferation and reversed his-
tone H3 hypermethylation induced by IDH2 R140Q expression (Supplementary Fig. S4A and S4B).

Treatment of TF-1 cells with erythropoietin (EPO) induces

Table 1. In vitro potency of AG-221 against IDH2-mutant, IDH2 WT, and IDH1 WT enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC50 (μmol/L) mean ± SD (max % inh) forward (oxidative)</th>
<th>IC50 (μmol/L) mean ± SD (max % inh) reverse (reductive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH2 WT homodimer + NADPH @ 1 h</td>
<td>39.83 ± 9.08*</td>
<td>0.32 ± 0.05 (99 ± 2)</td>
</tr>
<tr>
<td>IDH2 WT homodimer + NADP+ @ 1 h</td>
<td>1.80 ± 0.32 (88 ± 2)</td>
<td>0.31 ± 0.17 (96 ± 4)</td>
</tr>
<tr>
<td>IDH2 WT/R140Q heterodimer + NADP+/NADPH @ 1 h</td>
<td>0.38 ± 0.19 (73 ± 2)</td>
<td>0.18 ± 0.09 (71 ± 0)</td>
</tr>
<tr>
<td>IDH2 WT/R140Q heterodimer + NADP+ @ 16 h</td>
<td>0.04 ± 0.02 (75 ± 2)</td>
<td>0.03 ± 0.02 (89 ± 8)</td>
</tr>
<tr>
<td>IDH2 WT/R172K heterodimer + NADP+/NADPH @ 1 h</td>
<td>0.18 ± 0.09 (71 ± 0)</td>
<td>0.11 ± 0.01 (107 ± 4)</td>
</tr>
<tr>
<td>IDH2 WT/R172K heterodimer + NADP+ @ 16 h</td>
<td>0.03 ± 0.02 (64 ± 3)</td>
<td>0.01 ± 0.01 (100 ± 2)</td>
</tr>
<tr>
<td>IDH1 WT homodimer + NADP+ @ 1 h</td>
<td>1.12 ± 0.68 (77 ± 2)</td>
<td>77.64 ± 11.99*</td>
</tr>
<tr>
<td>IDH1 WT homodimer + NADP+ @ 16 h</td>
<td>0.45 ± 0.31 (75 ± 1)</td>
<td>48.40 ± 10.20*</td>
</tr>
</tbody>
</table>

NOTE: For activity against enzyme, the enzyme, cofactor, and compound were preincubated for 1 or 16 hours as described in Methods. For all enzyme assessments, n ≥ 3. The forward (oxidative) reaction refers to conversion of isocitrate and NADP+ to α-KG and NADPH, and the reverse (reductive) reaction to conversion of α-KG and NADPH to 2HG and NADP+.

Abbreviations: h, hour; inh, inhibitory; max, maximum.

*Consistent with the mechanism of action, IC50 measurements were carried out in the presence of NADP+ cofactor for the IDH1 WT and IDH2 WT homodimers; NADPH for the IDH2 WT/R140Q, IDH2 WT/R172K, and IDH1 WT/R132H homodimers; and a mixture of NADP+ and NADPH cofactors for the IDH2 WT/R140Q and IDH2 WT/R172K heterodimers.

†Fit to 100% (assay does not reach 100% inhibition at 100 μmol/L maximum compound concentration).

Table 2. In vitro potency of AG-221 for 2HG suppression

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell origin</th>
<th>Mutation</th>
<th>n</th>
<th>IC50 (μmol/L) mean ± SD (max % inh)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-116 Ki*</td>
<td>Human colorectal carcinoma</td>
<td>IDH2 WT/R140Q</td>
<td>9</td>
<td>0.53 ± 0.26 (84)</td>
</tr>
<tr>
<td>TF-1 pLVX*</td>
<td>Human erythroleukemia</td>
<td>IDH2 WT/R140Q</td>
<td>3</td>
<td>0.02 ± 0.01 (85)</td>
</tr>
<tr>
<td>TF-1 pLVX*</td>
<td>Human erythroleukemia</td>
<td>IDH2 WT/R172K</td>
<td>3</td>
<td>0.98 ± 0.18 (80)</td>
</tr>
<tr>
<td>U87MG pLVX*</td>
<td>Human glioblastoma</td>
<td>IDH2 WT/R172K</td>
<td>5</td>
<td>1.59 ± 0.42 (58)</td>
</tr>
<tr>
<td>U87MG pLVX*</td>
<td>Human glioblastoma</td>
<td>IDH2 WT/R140Q</td>
<td>9</td>
<td>0.01 ± 0.00 (96)</td>
</tr>
</tbody>
</table>

NOTE: Potency of AG-221 for 2HG suppression in cell lines with endogenous or ectopically expressed IDH2 WT/R140Q or IDH2 WT/R172K mutations was assessed based on 2HG levels in culture medium. Values are normalized to IDH2-mutant samples treated with DMSO (control).

Abbreviations: inh, inhibitory; max, maximum.

*Ectopic expression (knock-in mutation).

†Overexpression.
using fluorescence-activated cell sorting (FACS) for propidium iodide/Annexin V staining and cleaved caspase-3 or cleaved PARP protein expression (Supplementary Fig. SDD and SSE).

AG-221 also induced dose-dependent decreases in intracellular 2HG and cellular differentiation in ex vivo FACS-sorted primary blasts from patients with IDH2R140Q, or IDH2R172K-mutant AML. Consistent with the IC50 values estimated in cell lines (Table 2), primary AML samples expressing IDH2R140Q were more sensitive to the inhibitory action of AG-221 than those harboring the IDH2R172K mutation. When cultured in the presence of 0.1 μmol/L AG-221, IDH2R140Q cells showed an approximately 50% decrease in intracellular 2HG, whereas levels remained high in IDH2R172K cells (Fig. 2A). Consistent results were obtained when levels of 2HG in cell supernatant were considered. AG-221 treatment resulted in an increase in the percentage of cells expressing cell surface markers associated with granulocytic differentiation for both mutations, which was supported by cytology (Fig. 2B and C). Due to the heterogeneity of AML samples, several differentiation markers at several time points should be monitored in order to appreciate the responsiveness of IDH2-mutant cells to such inhibitors. Quantitative SNP assay PCR showed conservation of IDH2R140Q allele frequency following treatment in FACS-sorted mature myeloid cells (Fig. 2D), confirming that these mature cells were derived from IDH2-mutant blasts. We next evaluated the ability of AG-221 to promote the production of mature, functional neutrophils from IDH2-mutant blasts. Following culture in the presence of 5 μmol/L AG-221 for 8 days, IDH2R140Q blast cells (AML-8) exhibited granules colocalizing with lactoferrin (Fig. 2E), a canonical marker of secondary and tertiary granules (35), and a significantly higher number of cells with multilobed nuclei compared with control (mean ± SEM 32.4% ± 3.3% vs. 10.6% ± 1.8%; P < 0.001 by Student t test; Fig. 2F). Enhanced phagocytosis of opsonized latex beads was observed in AG-221–treated cultures on day 8 of treatment compared with control (mean ± SEM 66.5% ± 2.9% vs. 25.3% ± 9.3%; P < 0.001 by Student t test), illustrating the functional integrity of IDH2R140Q neutrophils (Fig. 2E and F). These data evidence a reprogramming of granulopoiesis upon AG-221 treatment, resulting in fully mature and functional neutrophils.

Together, these data indicate inhibition of 2HG production and reversal of downstream differentiation effects of the IDH2R140Q and IDH2R172K mutations.

**AG-221 Suppressed Production of 2HG in Tumor Xenograft Models**

To investigate the pharmacokinetic and pharmacodynamic effects of AG-221 on the extent and timing of 2HG suppression, we utilized a U87MG IDH2R140Q-mutant subcutaneous mouse xenograft model that exhibits high plasma and intratumoral 2HG concentrations (2.616 ± 86 ng/mL and 2.0 ± 0.10 × 10⁶ ng/g, respectively). AG-221 was administered to tumor-bearing mice as a single oral dose (25 or 50 mg/kg), and plasma and tumor concentrations of AG-221 and 2HG, respectively, were monitored over 3 days. AG-221 was detected throughout the study period, displaying rapid absorption and dose-proportional pharmacokinetics between 25 and 50 mg/kg, with a terminal half-life of 6 to 7 hours (Supplementary Fig. S6). 2HG reductions were observed 3 hours after dosing, with maximum reductions 12 hours after dosing of 93.3% and 96.2% in plasma (data not shown), and of 96.6% and 97.1% in tumors at 25 and 50 mg/kg, respectively (Supplementary Fig. S6). In response to decreasing plasma concentrations of AG-221, 2HG levels recovered with time, returning to predose levels 72 hours after dosing, when AG-221 plasma concentrations were < 5 ng/mL. 2HG reduction was even greater after 2 doses of 25 mg/kg given 12 hours apart (99.2% inhibition in tumors 8 hours after second dose), reaching levels observed in the plasma of WT mice (~200 ng/mL).

**AG-221 in Primary Human AML Xenograft Models**

To explore in vivo differentiation effects of AG-221, we established three xenograft models (AML-1, AML-2, AML-3) using freshly isolated, unsorted AML mononuclear cells from patients with IDH2R140Q-mutant AML (Supplementary Table S3). From each model, 10 mice with sustained human CD45-positive (hCD45) cell counts (range, 16%–66%) in bone marrow (BM) were randomly allocated to vehicle (n = 5) or AG-221 30 mg/kg twice daily (BID; n = 5) for 38 days.

The treatment was well tolerated: There were no abnormalities in body weight, hematoctrit, platelet counts, behavior, or food consumption in either group (Supplementary Fig. S7 and data not shown). Analyses of peripheral blood (PB) samples at multiple time points showed constant AG-221 serum concentration and near-normal 2HG levels in AG-221–treated animals (Supplementary Tables S4 and S5A), indicating effective inhibition of the IDH2R140Q-mutant enzyme. Intracellular 2HG levels also were reduced to below the limit of quantification in BM and spleen on day 38 of AG-221 treatment (Supplementary Table S5B). Upon AG-221 treatment, the hCD45+ subset of PB cells in models AML-1 and AML-2 acquired surface expression of several differentiation markers, including CD11b, CD14, CD15, and CD24 (Fig. 3A). The appearance of differentiated cells was observed between days 10 and 20 and reached >60% of the total human cell number by day 38.

In vehicle-treated mice, hCD45+ immunostaining at time of sacrifice (day 38) revealed large infiltrates of tightly packed positive cells in the BM and spleen, establishing that the human AML cells had homed to hematopoietic organs; these cells had also disseminated to nonhematopoietic tissues such as the liver, kidney, lung, and heart (Fig. 3B, Supplementary Fig. S8A, and data not shown). Hematoxylin–eosin–safranin (HES) staining showed a homogeneous population of immature cells, characterized by large hyperchromatic nuclei with detectable nucleoli (Supplementary Fig. SBB). In AG-221–treated mice, immunostaining on treatment day 38 revealed a dramatic decrease in hCD45+ cells in the BM (for models AML-1, AML-2, and AML-3 combined, mean ± SEM reduction of 71% ± 29%, spleen (reduction 50% ± 12%), and nonhematopoietic organs, and HES staining of the corresponding tissues displayed residual human cells with heterogeneous irregular shape and occasional pyknotic nuclei (Fig. 3B, Supplementary Fig. S8A and SBB, and data not shown). Accordingly, flow cytometry of BM-derived hCD45+ cells showed that AG-221 treatment induced an increase in cell granularity [indicated by an increase in side scatter (SSC)], the acquisition of CD14 or CD15 differentiation markers, and a decrease in
AG-221 can reduce intracellular 2HG levels and induce differentiation in primary human IDH2^R140Q- or IDH2^R172K-mutant AML patient samples treated ex vivo. Cells were cultured in the presence of AG-221 (0.1, 1, or 5 μmol/L) or DMSO (0.1%, v/v, used as a vehicle control) for up to 12 days. A, Percentage of intracellular 2HG remaining after 4, 8, and 12 days of treatment with AG-221 relative to DMSO control at the indicated doses. Data are represented as mean ± SEM of the % 2HG remaining (= 3 for each dose per genotype at days 4, 8, and 12). B, AG-221-induced fold changes in geometric mean fluorescence intensity (geo.MFI) for the CD24 or CD15 markers at day 8. C, Cytology analysis of May-Grünwald-Giemsa–stained BM-derived IDH2^R140Q-mutant cells (AML-3) showing granulocytic maturation on day 9 following treatment with 1 μmol/L AG-221. For the AG-221–treated cell population at day 9, differentiated cells were sorted by FACS according to expression of the CD24, CD15, and/or IDH2^R172K differentiation markers. Blue circles show allele frequency in IDH2^R140Q- and red triangles show allele frequency in IDH2^R172K-mutant allele frequency determined by quantitative SNP assay PCR using DNA from primary AML samples before culture (day 0) and at day 9 following treatment with 1 μmol/L AG-221. For the AG-221–treated cell population at day 9, differentiated cells were sorted by FACS according to expression of the CD24, CD15, and/or CD11b differentiation markers. Blue circles show allele frequency in IDH2^R140Q-AML samples (n = 3), and red triangles show allele frequency in IDH2^R172K-AML samples (n = 3). Results show that allele frequencies were all at approximately 50% in AML blasts carrying IDH2^R140Q and IDH2^R172K mutations in all of the indicated conditions. D, Confocal microscope images of primary AML cells treated with 5 μmol/L AG-221 (day 8), showing the presence of mature, functional neutrophils. Cells were stained for human lactoferrin (red), and DNA was labeled with DAPI (blue). Neutrophils were identified on the basis of morphologic characteristics (bilobed or multilobed nuclei). Phagocytic activity was assessed using opsonized latex beads (green). Data obtained from AML-8 are shown. Scale bars, 10 μm. E, Mature neutrophils (cells with multilobed nuclei), and neutrophils with phagocytic activity (cells with at least one latex bead phagocytized), were quantified in the AG-221– or control (DMSO)-treated cell populations. Data from AML-8 are shown as mean ± SEM. ***, P < 0.001 (Student t test).
Figure 3. AG-221 induces the differentiation of IDH2R140Q blasts along myeloid lineages in primary human AML xenograft models. A, Percentage of hCD45+ cells in the blood expressing any of the cell surface markers associated with differentiation along the monocytic/macrophage and granulocytic lineages (CD11b, CD14, CD15, or CD24) following treatment with AG-221 30 mg/kg b.i.d. or vehicle for 38 days, starting on day 0 (mean ± SD of combined data obtained from AML-1 and AML-2 is shown, n = 3 for both vehicle and AG-221). Statistical significance of AG-221–induced differentiation was determined using a two-way ANOVA with Bonferroni post-test corrections. *, P < 0.005 and **, P < 0.001. The AML-3 model was not included because it displayed very low levels of human cells in the blood; therefore, the effects of AG-221 on this parameter could not be assessed. B, hCD45 immunostaining (x100). In AG-221–treated mice, immunostaining revealed a dramatic decrease in hCD45+ cells in the BM (tibia). Representative samples from AML-2 shown. Scale bars, 100 μm. C, Cytology analysis of May-Grünwald-Giemsa–stained BM-derived cells illustrates the maturation of human blasts upon treatment with AG-221, as revealed by a decrease in the number of immature cells (blasts displaying cytoplasmic basophilia and high nucleocytoplasmic ratio [indicated by arrows on left plot]) accompanied by an increase in the number of more mature cell types such as myelocytes (black circles, right plot), metamyelocytes (black triangles, right plot), or neutrophils (black stars, right plot). Representative samples from AML-3 shown.
cells expressing the immaturity marker hCD117/c-KIT (Supplementary Fig. S9A and S9B). Cytologic analysis of BM-derived cells on treatment day 38 confirmed a 2- to 35-fold decrease in the percentage of human blasts upon AG-221 treatment, accompanied by an increase in the percentage of mature myeloid cells (Fig. 3C). Analyses of hCD45+ cells from the spleen also revealed AG-221-induced cell differentiation. Finally, the percentage of differentiated cells in the hCD45+ cell population in the BM was higher in AG-221-treated versus vehicle-treated mice, and quantitative SNP assay PCR confirmed the presence of the IDH2R140Q mutation (Supplementary Fig. S9C), demonstrating that AML blast cells, rather than cotransplanted normal human hematopoietic cells, had given rise to differentiated cells after in vivo AG-221 treatment, which supports a differentiation rather than a cytotoxic effect. These data show that AG-221 can dramatically reduce 2HG in the serum and BM, and reverse the IDH2R140Q-induced differentiation block in primary human AML xenograft models.

AG-221 Conferred a Dose-Dependent Survival Advantage in an Aggressive Human AML Xenograft Mouse Model

To determine if the in vivo differentiation effects of AG-221 were associated with a survival benefit, we established an aggressive human xenograft mouse model using early passage cells from a patient with AML harboring IDH2R140Q (AML-4; Supplementary Table S3). Once PB engraftment of hCD45+ AML cells reached approximately 10% (day 48 post-tail vein injection), animals were randomly allocated to vehicle or AG-221 at 5, 15, or 45 mg/kg once daily until end of treatment (day 84) or arabinofuranosyl cytidine (Ara-C) 2 mg/kg once daily for 5 days (Fig. 4A). AG-221 treatment was well tolerated and, compared with vehicle, conferred a dose-dependent survival advantage that was statistically significant at doses of 15 and 45 mg/kg; there was also a statistically significant survival advantage with AG-221 45 mg/kg versus low-dose Ara-C 2 mg/kg given for 5 days (P < 0.0001; Fig. 4B). Four animals in the AG-221 45 mg/kg group remained on treatment after day 84, and all survived until study termination (day 130).

This survival advantage was accompanied by reductions in 2HG levels and cell differentiation. As measured 8 hours after last dose (day 84), AG-221 exhibited a linear pharmacokinetic profile and effective inhibition of 2HG production in blood (89.7%, 91.9%, and 93.6%) and spleen (97.8%, 99.8, and 99.9%) at doses of 5, 15, and 45 mg/kg, respectively. In BM, 2HG levels were below the limit of quantitation in all AG-221–treated animals except for two in the lowest dose group (both 93.3% inhibition; Supplementary Table S6). On day 84, there was a dose-dependent decrease in the percentage of BM blast cells that was not seen in controls or mice treated with Ara-C for 5 days (Fig. 4C). Similar to the three less aggressive xenograft models (Supplementary Fig. S9C), the IDH2R140Q allele frequency in BM samples at termination was in the range of 37% to 52% across different AG-221 doses. Cytology showed the appearance of more mature differentiated myeloid cell forms compared with vehicle-treated mice, characterized by nuclear lateralization, coarse chromatim, and eosinophilic cytoplasm, in contrast to the decrease in cell size and nuclear fragmentation observed with Ara-C (Fig. 4D).

Finally, there was a dose-dependent increase in expression of CD15, a granulocytic marker of differentiation, in PB and BM of AG-221–treated mice that was not seen with Ara-C or vehicle (Fig. 5A and B). The lower percentage of differentiated cells seen in this model versus the less aggressive models likely reflects assessment here of only CD15+ cells within the CD45+ subset (additional differentiation markers were assessed in the other models). Interestingly, mice that died of leukemic disease in the 5 and 15 mg/kg dose groups (circled, Fig. 5A and B) failed to express CD15+, suggesting that onset of differentiation may be key to the survival of animals treated with AG-221.

DISCUSSION

There are limitations with currently approved chemotherapies for the treatment of AML, and although standard cytotoxic induction therapy is often effective initially, most patients relapse and become refractory, resulting in poor prognosis (36). IDH1/2 mutations can be identified in approximately 20% of patients with AML and approximately 5% of patients with MDS (37, 38), and can contribute to leukemia via a block in hematopoietic cell differentiation (16, 17, 23, 24, 39). We have discovered AG-221, an oral, selective, first-in-class inhibitor of the mutant IDH2 enzyme. AG-221 binds to an allosteric site within the dimer interface, stabilizing the open conformation of the enzyme and inhibiting the conversion of αKG to 2HG. AG-221 demonstrates excellent pharmaceutical properties, including adequate solubility, low clearance, and good oral bioavailability, and potently inhibits 2HG production by both the IDH2R140QWT heterodimer and IDH2R140Q homo-dimer. AG-221 shows noncompetitive inhibition with respect to the αKG substrate and uncompetitive inhibition with respect to the NADPH cofactor, and is a slow-on/slow-off inhibitor of the enzyme.

AG-221 exhibited robust 2HG suppression in multiple preclinical in vitro and in vivo IDH2R140Q-mutant systems, including primary human AML patient cells and xenograft mouse models of primary human AML, supporting its clinical development. In all models, 2HG suppression resulted in a release of the IDH2R140Q-induced cellular differentiation block, which in an aggressive AML xenograft mouse model was associated with a dose-dependent survival advantage versus vehicle that was statistically significant at higher doses. Survival was significantly better in the AG-221 45 mg/kg group compared with the 5-day, low-dose Ara-C group (P < 0.0001). Furthermore, with continuous AG-221 treatment, there was a dose-dependent decrease in the number of immature blasts observed in BM and an increase in the number of cells expressing differentiation markers in the BM and PB, supporting a differentiation effect. This was not seen in mice treated with Ara-C (2 mg/kg for 5 days) or in controls. Data showing that the IDH2-mutant allele frequency does not change upon AG-221 treatment indicate that mature cells were derived from IDH2-mutant blasts and support a differentiation effect. Although terminally differentiated myeloid cells undergo apoptosis, we hypothesize that, because the differentiation effect of AG-221 is slow relative to cytotoxic therapy, the percentage of terminally differentiated cells undergoing apoptosis at any given time point is low, and such cells would not be readily detectable. Our data from the
**Figure 4.** Effects of AG-221 treatment on survival and cell differentiation in an IDH2<sup>R140Q</sup> primary human AML xenograft model. 

**A.** Study design outlining random allocation of mice into dosing groups. 

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
<th>Dosing schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle q.d.</td>
<td>9</td>
<td>Continuously until death or study end</td>
</tr>
<tr>
<td>2</td>
<td>AG-221 5 mg/kg b.i.d.</td>
<td>9</td>
<td>Continuously until death or study end</td>
</tr>
<tr>
<td>3</td>
<td>AG-221 15 mg/kg b.i.d.</td>
<td>9</td>
<td>Continuously until death or study end</td>
</tr>
<tr>
<td>4</td>
<td>AG-221 45 mg/kg b.i.d.</td>
<td>9</td>
<td>Continuously until death or study end</td>
</tr>
<tr>
<td>5</td>
<td>Ara-C 2 mg/kg q.d.</td>
<td>4</td>
<td>5 days</td>
</tr>
</tbody>
</table>

**B.** Kaplan-Meier survival curves in mice treated with vehicle, AG-221, or low dose Ara-C. Dosing was initiated when tumor burden was approximately 10% in PB/60% in BM (day 48). At day 84, surviving mice were terminated except for four animals in the AG-221 45 mg/kg group, which remained on the same dose until study termination at day 130. 

- **AG-221 45 mg/kg b.i.d.**
- **AG-221 15 mg/kg b.i.d.**
- **AG-221 5 mg/kg b.i.d.**
- **Ara-C 2 mg/kg q.d.**
- **Vehicle**

**C.** Percentage of blasts in BM aspirates on last day of treatment (day 84); 200 cells counted per slide from representative mice in each treatment group. Horizontal bars represent the mean; P values for comparisons between treatment groups and vehicle were determined using the t test.

- P = 0.1624 × 10<sup>-17</sup> 
- P = 0.000015 
- P = 0.002 
- P = 0.0445 
- P = 0.1624 

**D.** BM morphology in vehicle, AG-221 45 mg/kg, and Ara-C 2 mg/kg groups. Wright-Giemsa stain, original magnification, ×1,000 oil. Representative results are shown.
TF-1 cell line support a lack of apoptosis induction by AG-221. In contrast to standard chemotherapy, the efficacy of AG-221 therefore appears to derive from induction of differentiation in malignant blasts. This mode of action results in an increase in myeloid differentiation, and production of mature, functional neutrophils, which may be advantageous to patients by avoiding the adverse effects of cytotoxic therapy (e.g., BM aplasia, susceptibility to severe infections, bleeding). In the aggressive AML xenograft mouse model, no changes in the variant allele frequency for other AML-related mutations were observed during AG-221 treatment; however, other patient-derived xenograft models may be suitable for monitoring possible differences in sensitivity of AML subclones to AG-221. Although our models focus on IDH2R140Q, in vitro studies indicated that AG-221 inhibits 2HG production by the IDH2WT/R172K heterodimer, and ex vivo studies demonstrated reduction in intracellular 2HG production and increase in expression of differentiation markers in primary human IDH2R172K-mutant AML patient samples, suggesting that AG-221 may also be effective against R172K-mutant tumors. Testing of primary in vivo AML models in addition to the four reported was not feasible in this study, owing to the difficulty in engrafting human IDH-mutant leukemia into mice.

The use of differentiation therapy in hematologic malignancies is exemplified by all-trans retinoic acid (ATRA) in acute promyelocytic leukemia (AML subtype M3), which induces blast proliferation followed by terminal differentiation and can induce short-term remission in approximately 85% of patients (40). Combination of ATRA with arsenic trioxide led to much-improved response rates, disease-free survival, and overall survival (40), highlighting the potential utility and synergy of combining differentiation therapy such as AG-221 with traditional chemotherapy or agents with other mechanisms of action, for the treatment of IDH-mutant tumors.

Preliminary data from a phase I trial indicated that AG-221 was well tolerated and had clinical activity in patients with relapsed/refractory AML, MDS, or untreated AML who declined conventional chemotherapy (41). These early results are promising and continue to validate mutant IDH as a therapeutic target. Further understanding of the role of IDH mutations in cancer initiation and progression will develop as the clinical data mature and as research broadens to additional mutant IDH-targeted molecules.

**METHODS**

**High-Throughput Screening**

Because the IDH2R140Q mutation confers a dramatic increase in affinity for NADPH (Km = 200 nmol/L; Supplementary Fig. S10A and S10B) relative to IDH2WT, we configured the screening assay at 10-fold concentration of Km for NADPH and at concentration of Km for αKG, to increase the likelihood of identifying NADPH-competitive and NADPH-noncompetitive inhibitors.

Potency (IC50 values) for lead compounds was assessed for the IDH2R172K-mutant homodimer in the presence of NADPH, as described below for AG-221. Cellular potency of lead compounds for 2HG suppression was carried out in a cell line with ectopically expressed IDH2R172K, based on 2HG levels in the culture medium (as detailed below for AG-221).

**Synthesis of AG-221**

AG-221 was prepared via a four-step process (Supplementary Fig. S1A), beginning with the condensation of methyl 6-(trifluoromethyl)picolinate with biuret to form the aryl substituted triazine-dione ring. Chlorination with phosphorous oxychloride yielded the dichlorotriazine. Displacement of the first chlorine with 4-amino-2-trifluoromethylpyridine generated the monochlorotriazine, which could be further reacted with 1-amino-2-methyl-2-propanol to produce AG-221 [2-Methyl-1-(4-(6-(trifluoromethyl)-pyridin-2-yl)-6-(2-

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**Figure 5.** Effects of AG-221 treatment on expression of CD15, a granulocytic marker of differentiation. Percentage of CD15+/CD45+ cells in the PB (A) and BM (B) of mice treated with vehicle, AG-221, or low-dose Ara-C, shown as mean ± SEM. Circles indicate mice that died of leukemic disease. q.d., once daily.
AG-221 Therapy for IDH2-Mutant AML

Expression and Purification of WT and Mutant IDH Enzymes

Proteins were expressed in bacterial or insect cell systems and purified by affinity chromatography. Details are provided in Supplementary Methods.

Determination of Compound Potency (IC50 Values)

AG-221 was prepared as 10 mmol/L stock in dimethyl sulfoxide (DMSO) and diluted to 50× final concentration in DMSO. IDH-mutant enzyme activity in converting αK to 2HG was measured in an end-point assay of NADPH depletion. In this assay, the remaining cofactor was measured at the end of the reaction period by the addition of a catalytic excess of diaphorase and resazurin to generate a fluorescent signal in proportion to the amount of NADPH remaining. IDH1R132 and IDH2R140 enzyme activity in converting isocitrate to αK was measured in a continuous assay directly coupling NADPH production to conversion of resazurin to resorufin by diaphorase. In both cases, resorufin was measured via fluorescence (λex = 544 nm, λem = 590 nm). IDHWT/mutant heterodimers were assayed for both WT and mutant activities. Details are provided in Supplementary Methods.

Cell-Based Assays for Measuring Inhibition of 2HG Production

The U87MG human astrocytoma (#HTB-14, ATCC; purchased 2009; not authenticated) and the TF-1 erythroleukemia (#CRL-2003, ATCC; purchased 2011; not authenticated) cell lines were infected with either pLVX-IDH2R140Q or pLVX-IDH2R172K, generated from the pLVX-IRES-Neo lentiviral vector (#632181, Clontech Laboratories, Inc.). TF-1 was verified to be growth factor-dependent in a proliferation assay against TF-1a cells (#CRL-2451, ATCC), a growth factor–independent erythroleukemia cell line derived from TF-1 cells. For both cell lines, characterization was carried out after plasmid infection: protein expression was assessed and 2HG levels were continuously monitored to verify authenticity of these overexpression lines. All transduced cell lines were selected and maintained in 500 µg/mL Geneticin in RPMI medium with 10% FBS and penicillin/streptomycin. The endogenous R172K-mutant HCT-116 cell line (HD104-019, Horizon Discovery Group) was purchased in 2013 (not authenticated), and intracellular 2HG levels were assessed to verify IDH2-mutant status.

In order to test the potency of AG-221, cells expressing either IDH2R140Q or IDH2R172K were plated in 96-well microtiter plates overnight at 37°C in 5% CO2. Compounds were plated in dose response in two columns to generate a seven-point dose response in duplicate. Doses were usually started at 3 mmol/L with 1:3 or 1:10 dilutions. AG-221 was diluted in DMSO to a final concentration of 0.03% DMSO in media. One row of 10 wells was designated for the 0.03% DMSO control. Cells were incubated with compound for 48 hours. Media were removed and 2HG was extracted using 80% aqueous methanol, as previously described, and the measurement of 2HG was expressed as ng/mL in medium (the lower limit of quantification was 10 ng/mL and the upper limit of quantification was 30,000 ng/mL). The data were normalized to the DMSO controls to express percent 2HG suppression as follows: (DMSO 2HG – inhibitor 2HG)/DMSO 2HG. The percent inhibition values were then plotted against the log of the dose. A sigmoidal dose-response equation using a variable slope (four parameters) was used to generate the IC50 for 2HG suppression as follows: (DMSO 2HG – inhibitor 2HG)/(DMSO 2HG) = 1 / [1 + ((IC50/log(inhibitor)) versus response−variable slope (four parameters)]. The data were expressed as IC50 for 2HG suppression (17).

Protein Purification for X-ray Crystallography Studies

His-tagged protein for co-crystallization of AG-221 was expressed in Sf9 insect cells (#CRL-1711, ATCC) and purified via immobilized metal affinity chromatography, then DEAE and size-exclusion chromatography. Protein for co-crystallization with αK was expressed in E. coli and purified as above. Details are provided in Supplementary Methods.

Crystallization, Data Collection, and Structure Determination

AG-221 co-complex crystals were generated by incubating IDH2R140Q purified from insect cells at 15 mg/mL with 10 mmol/L NADPH and 2 mmol/L of AG-221 or αK at 4°C for 1 hour. Co-crystals of the complex were grown by hanging drop vapor diffusion technique (HDVD) equilibrating the above mixture with a reservoir solution containing 0.1 mol/L Tris-HCl pH 8.5, 0.25 mol/L CaCl2, and 25% PEG4000 in a 1:1 ratio at 18°C. For the αK complex, IDH2R140Q purified from E. coli at 15 mg/mL was incubated with 5 mmol/L NADPH, 50 mmol/L of αK, and 5 mmol/L CaCl2 at 4°C overnight. Co-complex crystals were grown by HDVD equilibrating the mixture with a reservoir solution containing 200 mmol/L magnesium acetate, 100 mmol/L sodium cacodylate, pH 6.5, and 20% PEG3000 in a 1:1 ratio at 18°C.

Crystals were flash-frozen in liquid nitrogen before data collection after equilibrating them in a buffer containing reservoir solution and 20% (v/v) glycerol as a cryoprotectant.

X-ray characterization and data collection for both co-complexes were performed at the Shanghai Synchrotron Radiation Facility. Diffraction data were processed using HKL2000 (HKL Research Inc.; ref. 42). Crystals were characterized to be of orthorhombic form with space grouping of either C2221 or P212121. Statistics of data collection, processing, and refinement are summarized in Supplementary Table S7. The structure was determined by molecular replacement with Phaser (43) using the structure of IDH2 (Protein Data Bank ID: 4JAB) as a search model. Iterative manual model building and refinement were carried out using COOT (44) and Refmac5 (45) from the CCP4 package. Examination of difference Fourier map (Fo−Fc) calculations clearly indicated the presence of bound ligands. The electron density maps, contoured around the bound ligands, are shown in Supplementary Fig. S11A and S11B. Two alternative poses were fit for the AG-221 molecule, with the occupancy for each pose modeled at 0.6 and 0.4 in Chains A and B, respectively. Consistent with the ligand binding mode, multiple residues in the binding pocket surrounding AG-221 were also modeled to fit alternative conformations. The final Rwork/Rfree for IDH2R140Q-AG-221 and IDH2R140Q-αK structures are 0.153/0.194 and 0.142/0.174, respectively. Structure figures were generated using MOE (Chemical Computing Group, Inc.) and PyMOL (Schrödinger, LLC). Amino coordinates and experimental structure factors have been deposited at the RCSB Protein Data Bank with accession codes 5I95 for the IDH2R140Q-αK and 5196 for the IDH2R140Q-AG-221 complex structures.

TF-1 Cell Experiments

TF-1 cell experiments were conducted as previously reported (17).

Human IDH2-Mutant AML Samples

All primary IDH2-mutant patient AML samples, except AML-4, were provided by Gustave Roussy (Department of Clinical Hematology), according to Institutional Review Board–approved protocols. Informed consent was obtained from all patients, in accordance with the Declaration of Helsinki. AML diagnosis was morphologically proven according to the French–American–British classification. Immunophenotyping and cytogenetic analyses were done locally. Description of the karyotypes follows the International System for Human Cytogenetic Nomenclature. Samples were obtained from PB and/or BM aspirates from patients with IDH2R140Q-mutant AML at diagnosis or at relapse. Mononuclear cells were isolated by Ficoll separation. Mutation status was assessed by performing targeted
sequencing of 60 genes frequently mutated in hematologic malignancies using a MiSeq sequencer (Illumina) and a custom panel primer pool, using methods described previously (46, 47). We selected variations using the following criteria: variant allele frequency >10%, variation not reported as polymorphism in SNP databases.

AML-4 corresponds to a human cell line, AMM7577, which was obtained with informed consent from the BM of a male patient with M5 AML who had relapsed and died at 59 years of age. This cell line has a normal karyotype and carries IDH2R140Q, FLT3-ITD, DNMT3A882fs, NPM1, and CEBPA insertion.

The clinical characteristics of these samples are indicated in Supplementary Table S3.

Ex Vivo Cell Culture

Nine AML samples [IDH2R140Q, n = 3 (AML-3, -5, -6); IDH2R172K, n = 3 (AML-7, -8, -9); IDH2WT, n = 3 (AML-10, -11, -12)] were used, containing more than 70% blast cells, except AML-7. For the latter, leukemic blasts identified as CD45int/SSClow cells (cells were stained with anti-human CD45 antibody PE-Cy7–hCD45, clone HI30; BD Biosciences) were sorted using a BD Influx Cell Sorter (BD Biosciences). The clinical characteristics of these samples are indicated in Supplementary Table S3. Leukemic cells were cultured in the presence of AG-221 (0.1, 1, or 5 μmol/L) or DMSO (0.1%, v/v, used as a vehicle control) for up to 12 days in StemSpan culture medium supplemented with 0.5% HyClone FCS, human IL3, IL6, stem cell factor, thrombopoietin, EPO, FMS-like tyrosine kinase 3 ligand, granulocyte-macrophage colony-stimulating factor, and granulocyte-colony stimulating factor (all from PeproTech) to sustain cell survival and proliferation, as reported (17). Cell samples were collected at day 8, and flow cytometry analyses were performed to determine phenotype. Antibodies used were as follows (all 1 µg/mL final concentration): PE-Cy7–CD45 (clone HI30), PE–CD11b (clone ICRF44), APC-Cy7–CD14 (clone Mp9), eFluor450–CD15 (clone H98), and APC–CD24 (clone ML5; all from BD Pharmingen). Intracellular and extracellular levels of D-2HG were determined as previously described (48).

Phagocytosis Assay

Phagocytosis tests were performed with opsonized latex beads as previously reported (49). Briefly, on day 8 of AG-221 treatment, cells were collected and resuspended in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10 mmol/L HEPES and 10% heat-inactivated human serum (Invitrogen) to a final concentration of 106 cells/mL. Cells were incubated on ø12 mm coverslips in 24-well plates with ø1 mm fluorescent latex beads (Invitrogen), applied at a ratio of approximately 10 beads/cell. After 10-minute centrifugation at 300 g, cells were incubated for 30 minutes at 37°C to initiate phagocytosis prior to fixation in a paraformaldehyde 4% solution (Sigma-Aldrich). Fixed cells were labeled with 4′,6-diamidino-2-phenylindole (DAPI). Labeled cells were imaged with a Leica DMI5000 B fluorescence microscope (Leica). Neutrophils were identified on the basis of morphologic characteristics (biloced or multiloced nuclei), and neutrophils with phagocytic activity, defined as neutrophils containing at least one phagocytized bead, were scored in ten fields of view, on approximately 300 total cells. Schematic representations and statistical analyses were performed with the Prism 7 software (GraphPad Software, Inc.).

Mouse Husbandry

All mouse experiments were approved by and performed in accordance with the guidelines and regulations of the Animal Ethics Committee of the Association for Assessment and Accreditation of Laboratory Animal Care International. AML-1, AML-2, and AML-3 mice were housed under pathogen-free conditions in micro-isolator cages at the animal facilities of Crown Bioscience, Inc.

Pharmacokinetic/Pharmacodynamic Study of AG-221 in the U87MG IDH2R140Q Xenograft Model

AG-221 was suspended in 0.5% methyl cellulose and 0.2% Tween 80 in water and given as a single dose of 25 mg/kg or 50 mg/kg, or as two doses of 25 mg/kg 12 hours apart, to 11-week-old female BALB/c nude mice (BK Laboratory Animal Ltd.) with U87MG IDH2R140Q xenograft tumors. A separate group of mice was dosed with the suspension vehicle. Groups of 4 mice were sacrificed at pre-dose, 0.5, 1, 3, 8, 12, 24, 36, 48, and 72 hours after dose to collect tumor samples and blood for plasma analysis. AG-221 and 2HG levels were analyzed by LC/MS-MS.

Primary IDH2R140Q AML Xenotransplantation and AG-221 Treatment

Clinical characteristics and immunophenotypic features of the patients who provided samples to develop these xenograft models are reported in Supplementary Table S3.

For AML-1, AML-2, and AML-3 samples, unsorted AML mononuclear cells (106) were transplanted into adult (8–10 weeks old), female, sublethally irradiated (2 Gy) NOD/SCID IL2Rγc−/− (NSG) mice by intraperitoneal injection. NSG mice were maintained in pathogen-free conditions. The presence of hCD45+ cells in BM and in PB was monitored on a monthly basis by flow cytometry using the PE-Cy7–hCD45 antibody (clone HI30; BD Biosciences) on a BD LSRII flow cytometer (BD Biosciences). Engrafted recipients, assessed by the presence of ≥16% hCD45+ cells in BM, were randomly selected for treatment with either AG-221 30 mg/kg (n = 5) or vehicle solution (n = 5). Investigators were not blinded to treatment group assignment.

AG-221 mesylate powder was resuspended by sonication in 6 mg/mL of vehicle solution composed of 0.5% methylcellulose/0.2% Tween 80 diluted in water. Animals were treated b.i.d. by oral gavage for 38 days. For the AML-4 sample, a total of 50 female 3- to 4-week-old NOD/SCID mice (Beijing HFK Bioscience Co., Ltd.) were engrafted with 2 million frozen cells per mouse (AMM7577 passage 2). PB samples were collected by retro-orbital blood weekly for FACS analysis starting from week 3, after cell inoculation. Treatment started when the percentage of hCD45+ cells in PB reached an average of 10% of total white blood cells. Mice were randomly allocated to one of five groups and treated with vehicle once daily continuously (group 1), AG-221 at 5, 15, or 45 mg/kg b.i.d. continuously until death or study end (day 84; groups 2–4), or low-dose Ara-C at 2 mg/kg once daily for 5 days (group 5). This was a nonblinded study. The Ara-C dose of 2 mg/kg was selected based on dosing in previous leukemia xenograft mouse models (50) and preliminary experiments using doses of 2 and 10 mg/kg in this model, in which similar efficacy was observed for both doses. The 5-day dosing schedule was intended to mimic the standard 7+3 regimen used in the treatment of AML. We were unable to treat animals with low-dose Ara-C for longer due to the toxicity of this agent in these mice. Treatment was by oral gavage (at 12-hour intervals for AG-221). At day 84, all surviving mice were terminated except for four animals in the AG-221 45 mg/kg group, which remained on the same dose until study termination at day 130 in order to further assess biology and determine if survival was extended.

FACS Analysis of PB, BM, and Spleen Samples

Blood (50 μL) was collected into EDTA tubes by retro-orbital bleeding. Nine volumes of red blood cell lysing buffer (0.8% ammonium chloride solution; Stemcell Technologies) were added to each tube and incubated on ice for 5 minutes. Samples were centrifuged for 5 minutes at 1,500 rpm at 4°C. The supernatant was discarded and cells resuspended with PBS containing 2% FBS. Multicolor FACS analysis was carried out on PB samples using the following antibodies for conditions in micro-isolator cages at the animal facilities of Crown Bioscience, Inc.
models AML-1, AML-2, and AML-3: PE-Cy7–CD45 (clone H130; eBioscience SAS), FITC–CD14 (clone McPh9; BD Pharmingen), PE–CD11b (clone ICRF44; BD Pharmingen), eFluor450–CD15 (clone H198, eBioscience), and APC–CD24 (clone MLS; BD Pharmingen). Percentages of human chimerism in blood are indicated in Supplementary Table S8. At sacrifice, human cells were immunohistochemically identified as single-cell suspensions from PB, spleen, and BM (mixed from tibia, femurs, pelvic bones, and humerus), after lysis of red blood cells with 0.8% ammonium chloride solution (Stemcell Technologies) using the above-mentioned antibodies as well as PE–CD3 (clone HIT3a; BD Pharmingen), PE-Cy7–CD45 (clone H130; eBioscience SAS), PE-Cy7–CD34 (clone 4H11; eBioscience), and PE–CD117 (c-Kit, clone 104D2; BD Pharmingen). For model AML-4, the following antibodies were used: CD45 (clone H130; BioLegend) and CD15 (clone W6D3; BioLegend). Data were collected with the BD LSR II system using FACSDiva software (BD Biosciences) and with the BD FACSCalibur system using CellQuest 6 software (BD Biosciences) and analyzed using FlowJo version 9.3.1 software (Tree Star, Inc.).

Histology

The 4% paraformaldehyde-fixed, paraffin-embedded, 4-μm tissue sections were analyzed by HES staining and by standard immunohistochemistry using the anti-hCD45 antibody (clone 2B11+PD7/26; DakoCytomation) or nonspecific IgG1 (DakoCytomation). Images were taken at 100x and 400x magnification using an Axiohot microscope (Zeiss) coupled to a sensaica 12-bit cooled imaging camera (PCO AG). Morphologic cytology analyses were performed on BM and blood cells after May-Grünwald-Giemsa staining. Image analysis was performed using Definiens Composer software. Regions to be analyzed were first drawn manually and then refined based on automated pattern recognition to separate them into three classes: tumor (hCD45+ cells), white, and normal cells. Percentage inhibition was calculated as follows: ratio = (% hCD45-positive area) / (% hCD45-positive area + % normal cell area). % h = (ratio vehicle – ratio AG-221)/ratio veh x 100.

IDH2 Mutant Allele Frequency

Determination of IDH2-mutant allele frequency was carried out by extracting DNA from primary IDH2R140Q and IDH2R172K AML samples before culture, or cells were cultured in the presence of vehicle or 1 μmol/L AG-221 for 9 days, and used as a template for quantitative SNP assay PCR as described (17). At day 9, differentiated cells were sorted by FACS on a BD Influx Cell Sorter (BD Biosciences) according to the expression of differentiation markers CD24, CD15, and/or CD11b. In in vivo experiments, DNA was extracted from sorted BM or spleen samples from AML-1-, AML-2-, and AML-3-engrafted mice treated with AG-221 for 38 days.

Statistical Analyses

No formal sample-size calculations were performed in the xenograft model treatment studies. For the Kaplan-Meier survival curve, statistical significance was determined by the log-rank (Mantel-Cox) test using the survival package in CRAN.

Data Deposition Statement

Atomic coordinates and experimental structure factors have been deposited at the RCSB Protein Data Bank with accession codes S195 for the IDH2R140QkoKG and S196 for the IDH2R172KAG-221 complex structures.

Disclosure of Potential Conflicts of Interest

J. Travins has ownership interest (including patents) in Agios Pharmaceuticals. K. Straley has ownership interest in Agios stock. S. Gross has ownership interest (including patents) in Agios Pharmaceuticals. F.G. Salituro has ownership interest in Agios stock and patent applications. S.A. Biller has ownership interest (including patents) in Agios Pharmaceuticals. S.-S.M. Su has ownership interest (including patents) in Agios Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Yen, F. Wang, M.D. David, E. Artin, K. Straley, S. Gross, B. DeLaBarre, Y. Chen, R. Nagaraja, C. Quivoron, P. Opolon, V. Saada, S. Broutin, S. de Botton, B.S. Marteyn, F. Jiang, H. Yang, V. Penard-Lacroix


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Yen, F. Wang, J.O. Saunders, O. Bawa, V. Saada, S. Jin

Study supervision: K. Yen, F. Wang, R. Nagaraja, O.A. Bernard, V. Penard-Lacroix, S.A. Biller

Other (image submission): P. Opolon

Other (produced the protein to support assay development): C. Fang

Other (designed research program): S.-S.M. Su

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