ORIGINAL ARTICLE

Pan-mutant-IDH1 inhibitor BAY1436032 is highly effective against human IDH1 mutant acute myeloid leukemia *in vivo*

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Neomorphic mutations in isocitrate dehydrogenase 1 (*IDH1*) are frequently found in several human cancer types including acute myeloid leukemia (AML) and lead to the production of high levels of the oncometabolite (*R*)-2-hydroxyglutarate (R-2HG). Here we report the characterization of BAY1436032, a novel pan-mutant IDH1 inhibitor, both *in vitro* and *in vivo*. BAY1436032 specifically inhibits R-2HG production and colony growth, and induces myeloid differentiation of AML cells carrying IDH1R132H, IDH1R132C, IDH1R132G, IDH1R132L and IDH1R132S mutations. In addition, the compound impacts on DNA methylation and attenuates histone hypermethylation. Oral administration of BAY1436032 led to leukemic blast clearance, myeloid differentiation, depletion of leukemic stem cells and prolonged survival in two independent patient-derived xenograft IDH1 mutant AML mouse models. Together, BAY1436032 is highly effective against all major types of IDH1 mutant AML.

INTRODUCTION

Isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate (αKG). Mutations in isocitrate dehydrogenase 1 (*IDH1*) and *IDH2* were found in several tumors including glioma,[1,2] acute myeloid leukemia (AML),[3] myeloproliferative neoplasm[4] and myelodysplastic syndrome patients,[5] chondrosarcoma,[6] lymphoma,[7] melanoma[8] and thyroid cancer.[9] Mutations in *IDH1* mostly occur at arginine 132, with substitutions including R132H, R132C, R132G, R132L and R132S, while mutations in *IDH2* occur at arginine 172 or arginine 140[(refs 10–12)] and affect the active sites, where IDH1/2 substrates isocitrate and NADP$^+$ bind.[13–16] Mutant IDH loses its normal activity with concomitant gain of a neomorphic function that catalyzes conversion of αKG to the oncometabolite (*R*)-2-hydroxyglutarate (R-2HG), which induces histone- and DNA hypermethylation through inhibition of demethylation, and leads to a block in cellular differentiation promoting tumorigenesis.[17–22] The prognostic significance of *IDH1* mutations in AML remains controversial, with a preponderance of studies suggesting that mutant IDH1 confers an adverse prognosis or has no prognostic value.[23] Serum R-2HG concentration may also serve as a prognostic indicator.[24–26]

AML treatment has not been significantly improved for the past four decades with the majority of patients eventually relapsing and dying of the disease. In cytogenetically normal AML, mutated *IDH1* is found in 10.9% of patients,[11] while mutated *IDH2* is found in 12.1%.[10] Recently, it has been shown that R-2HG, the oncometabolite produced by mutant IDH, promotes leukemogenesis even in the absence of mutant IDH and that pretreatment R-2HG serum levels impact on outcome in IDH1 mutant AML.[21,24] Previous studies have revealed that the mutant IDH enzyme remains important for the growth of IDH mutant cancers once they are fully established, and treatment with a mutant selective inhibitor induces cellular differentiation *ex vivo*.[21,27] We have also previously shown that pharmacological inhibition of the mutant IDH1 enzyme blocks colony formation of human AML cells but not of normal CD34$^+$ bone marrow cells *in vitro*.[28] Recently, a novel IDH1 inhibitor reduced 2-HG levels and induced differentiation *in vivo* after intraperitoneal administration in mice.[29] However, no beneficial effect of IDH1 inhibitors on survival of mice has been reported so far. The first clinical inhibitor of mutant IDH1, AG-120, induced complete remission in 18% and an overall response in 36% of patients.[30] An initial report of AG-120 treated patients showed that AML blasts differentiate to mature myeloid cells, but the allele burden of mutant IDH1 remained high in a considerable number of patients.[30] This suggests that inhibition of mutant IDH1 induces differentiation, but may not deplete leukemic stem cells *in vivo*. We developed a novel, highly active oral pan-IDH1 inhibitor, BAY1436032, and evaluated its preclinical activity in two independent patient-derived xenograft (PDX) AML mouse models. We show that BAY1436032 depletes AML cells in peripheral blood,

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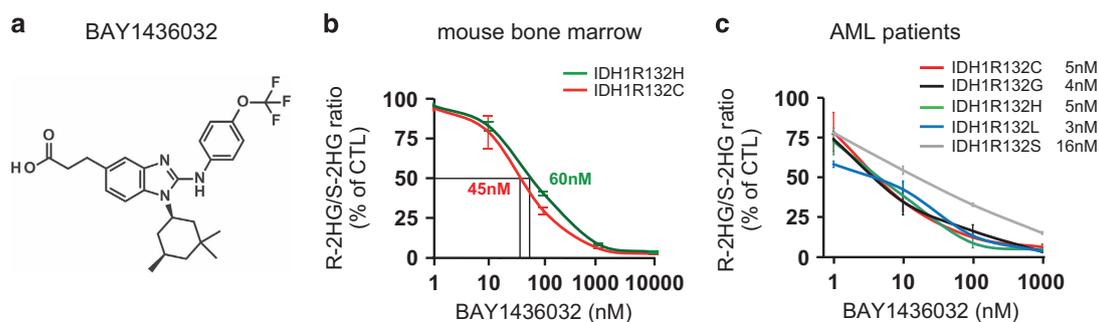
Figure 1. BAY1436032 selectively inhibits R-2HG production in IDH1 mutant mouse hematopoietic and primary human AML cells. (**a**) Chemical structure of BAY1436032. (**b**) Ratio of R-2HG to S-2-hydroxyglutarate (S-2HG) after 8 days of BAY1436032 treatment of HoxA9-immortalised mouse bone marrow cells retrovirally transduced with IDH1R132C or IDH1R132H. Half maximal inhibitory concentration (IC$_{50}$) values were calculated as percentage of dimethyl sulfoxide (DMSO; CTL) treatment and are indicated in the graph (mean ± s.e.m., $n = 3$). (**c**) Ratio of R-2HG to S-2HG in primary human AML cells harboring different IDH1 mutations 24 h after BAY1436032 treatment. IC$_{50}$ values were calculated as percentage of DMSO (CTL) treatment and are given in the graph (mean ± s.e.m., $n = 3$).

prolongs survival, and inhibits leukemia stem cell (LSC) self-renewal *in vivo*.

MATERIALS AND METHODS

Cell culture and treatment

HoxA9-IDH1R132H, HoxA9-IDH1R132C, HoxA9-IDH2R140Q and HoxA9-IDH2172K cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum (FBS), 10 ng/ml of human interleukin 6 (hIL-6), 6 ng/ml of murine interleukin 3 (mIL-3), and 20 ng/ml of murine stem cell factor (mSCF; all from PeproTech, Hamburg, Germany) and were incubated at 37 °C with 5% CO$_2$ in humidified atmosphere. Patient-derived AML cells, freshly isolated or cryopreserved, were cultured in IMDM medium (Gibco, Karlsruhe, Germany) supplemented with 12.5% FBS (Gibco), 12.5% horse serum (Gibco), 5 μM hydrocortisone, 2.5 mM GlutaMax, 10 ng/ml human FLT3-ligand, 10 ng/ml human TPO, 50 ng/μl human SCF and 10 ng/μl human IL-3 (all from PeproTech) and were incubated at 37 °C with 5% CO$_2$ in humidified atmosphere. Treatment was carried out with BAY1436032 or dimethyl sulfoxide for indicated time points and concentrations.

Clonogenic progenitor assay

Colony-forming cell (CFC) units were assayed in methylcellulose (Methocult H4100; StemCell Technologies Inc., Cologne, Germany) supplemented with 10 ng/ml IL-3, 10 ng/ml GM-CSF, 50 ng/ml SCF, 50 ng/ml FLT3-ligand and 3 U/ml EPO (PeproTech). Vehicle or BAY1436032 was added to methylcellulose containing 10^5 human mononuclear cells, which were plated in duplicate. Colonies were evaluated microscopically 10 to 14 days after plating by standard criteria.

Antibodies for flow cytometry

Monoclonal antibodies used were CD45-FITC (2D1), CD45-PerCP-Cy5.5 (HI30), CD34-APC (8G12), CD34-FITC (HIT2), CD38-PE (HB-7), CD33-PE (WM53), CD14-APC (M5E2), CD14-FITC (M5E2), CD15-PE (W6D3), CD3-PE (UCHT1), CD19-APC (HIB19) from BD Biosciences (Heidelberg, Germany) and CD15-PE (80H5) from Beckman Coulter (Krefeld, Germany). Data were collected on either an Accuri C6 or a FACS Calibur (BD Biosciences).

RESULTS

On-target activity of BAY1436032, a novel IDH1 inhibitor, in IDH1-mutant hematopoietic cells

We developed a novel IDH1 inhibitor, BAY1436032, with high selectivity against all known IDH1R132 mutant proteins (R132H, R132C, R132G, R132S and R132L) compared to wild-type IDH1 and wild-type or mutant IDH2.[31] Details on chemical development, structural characteristics (Figure 1a) and activity profiles towards recombinant mutant and wild-type IDH1 and IDH2 proteins of BAY1436032 have been described by Pusch *et al.*[31] To assess the inhibitory effect of BAY1436032 on the enzymatic function

of mutant IDH1 in a hematopoietic cell context, we employed immortalized mouse hematopoietic cells retrovirally engineered to express mutant IDH1 or IDH2, and primary human IDH1 or IDH2 mutant cells derived from AML patients. BAY1436032 inhibited intracellular R-2HG production in mouse hematopoietic cells expressing IDH1R132H or IDH1R132C with an IC$_{50}$ of 60 and 45 nM, respectively (Figure 1b). In contrast, R-2HG levels were not reduced in IDH2R140Q and IDH2R172K expressing mouse hematopoietic cells by BAY1436032 at concentrations up to 10 μM (Supplementary Figure 1A). Patient-derived AML cells harboring IDH1R132C, IDH1R132G, IDH1R132H, IDH1R132L, or IDH1R132S mutations were very sensitive to BAY1436032 *ex vivo* with IC$_{50}$ values between 3 and 16 nM whereas the compound had virtually no effect upon patient-derived AML cells with IDH2R140Q or IDH2R172K mutations at concentrations up to 1 μM (Figure 1c and Supplementary Figure 1B). Thus, BAY1436032 displays on-target activity towards mutant IDH1 in both mouse and primary human hematopoietic cells.

BAY1436032 inhibits proliferation and induces differentiation in primary human AML cells

Next, we assessed the effect of BAY1436032 on proliferation and differentiation of primary human AML cells with wild-type or mutant IDH1. Patient-derived AML cells harboring either wild-type IDH1 or IDH1R132H, IDH1R132C, IDH1R132L or IDH1R132S mutations were seeded in semi-solid medium supplemented with BAY1436032 at different concentrations or vehicle. Colony growth was inhibited by 50% at a concentration of 0.1 μM BAY1436032, while concentrations up to 100 μM did not suppress colony growth of patient-derived IDH1 wild-type AML cells (Figure 2a). IDH1 mutant AML cells cultured in suspension medium *ex vivo* showed marked upregulation of myeloid differentiation markers CD14 and CD15 (Figure 2b and Supplementary Figure 1C). On morphologic evaluation myelomonocytic differentiation of myeloid progenitors was strongly induced by BAY1436032 (Figure 2c). These data suggest that BAY1436032 inhibits proliferation and induces differentiation of primary IDH1 mutant AML cells *ex vivo*. As primary AML cells can only be propagated in culture for a limited time before spontaneous apoptosis occurs, we aimed to validate these findings *in vivo* in PDX AML mouse models.

Favorable pharmacokinetics allow once daily dosing of BAY1436032

A PDX mouse model was developed using primary AML cells from a patient with IDH1R132C mutant AML (PDX1). Targeted sequencing of the patient AML cells revealed a *FLT3*-TKD (p.D835del), an atypical *NPM1* (p.S254LfsTer4), and a *NRAS* (p.Q61R) mutation as
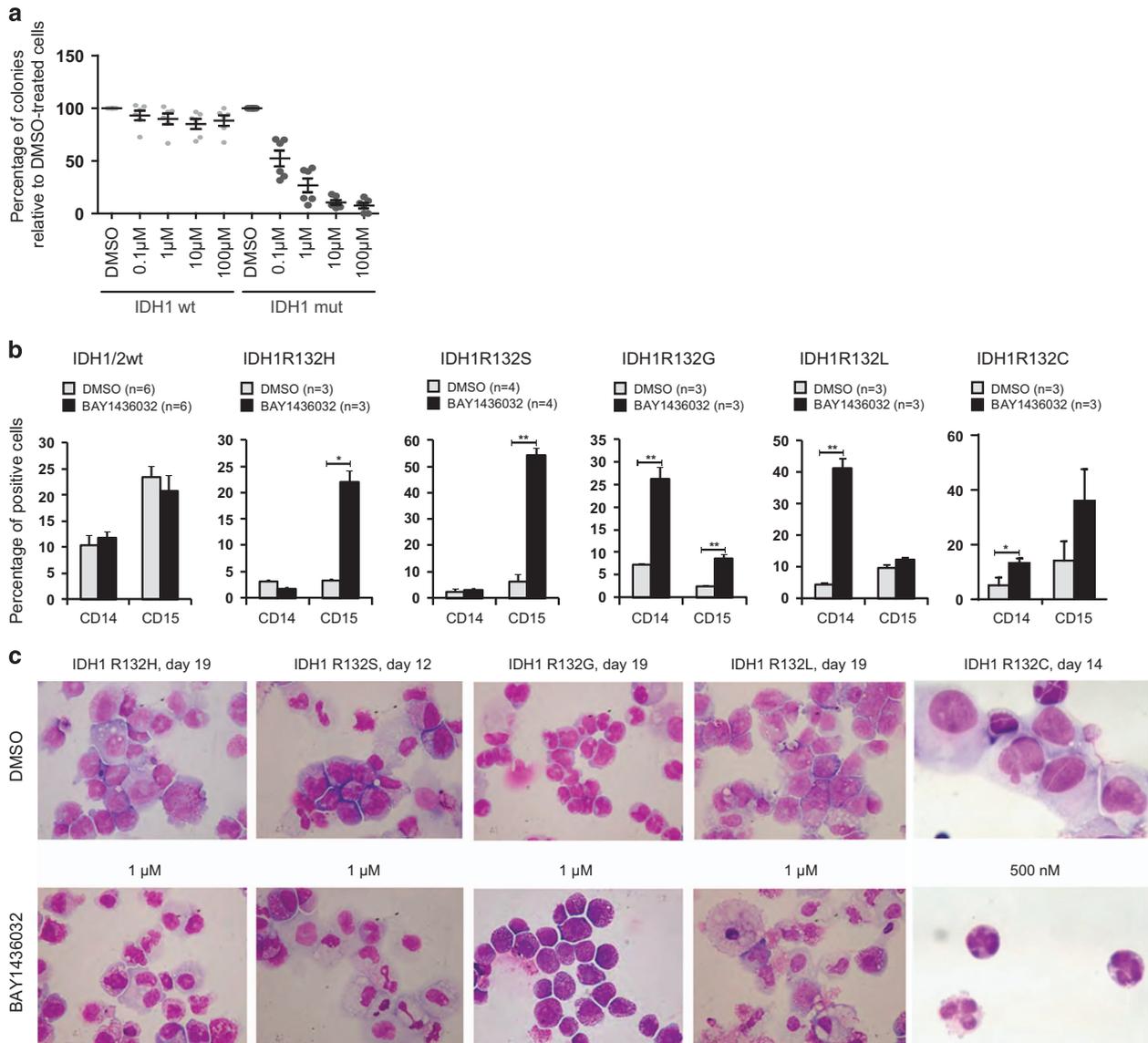
Figure 2. BAY1436032 inhibits proliferation and induces myeloid differentiation in patient-derived IDH1 mutant AML cells *ex vivo*. (**a**) Inhibition of colony formation by BAY1436032 in colony-forming cell assays using primary human AML cells with wild-type (wt) or mutant (mut) IDH1 (mean ± s.e.m., *n* = 6). From the six patients with IDH1 mutant AML, three harbored a IDH1R132H mutation and one each IDH1R132C, IDH1R132L and IDH1R132S mutations, respectively. (**b**) Quantification of IDH1 wild-type (wt) or IDH1 mutant primary AML cells expressing CD14 and CD15 after *ex vivo* treatment with dimethyl sulfoxide (DMSO) or BAY1436032 (mean ± s.e.m.). Numbers of patient samples examined are given in the graph. *$P < 0.05$; **$P < 0.01$. (**c**) Morphology of primary IDH1 mutant AML cells after *ex vivo* treatment with DMSO or BAY1436032 showing signs of monocytic/granulocytic maturation (×1000 or ×4000 magnification). Treatment durations and BAY1436032 concentrations are given in the graph.

additional aberrations. These cells were propagated in primary NSG recipient mice and upon stable engraftment retransplanted into secondary recipients, where the mutations initially found could be confirmed. The cells were then transplanted into tertiary recipients, which were used for treatment with BAY1436032. Plasma exposure of BAY1436032 was almost dose-linear between 45 and 150 mg/kg with unbound concentrations covering the *in vitro* R-2HG/S-2HG ratio IC$_{50}$ for 24 h (Supplementary Figure 2A). At all tested doses of BAY1436032 R-2HG serum levels declined rapidly starting as early as three hours after application, reaching 5-, 6- and 7.5-fold reductions with 45, 90 and 150 mg/kg BAY1436032, respectively, after 24 h (Supplementary Figure 2B). Long-term exposure to once daily oral BAY1436032 revealed nearly complete suppression of R-2HG production with 150 mg/kg

BAY1436032 (Supplementary Figures 2C and D). Therefore, the pharmaco kinetic profile allowed once daily oral dosing for subsequent PDX *in vivo* experiments (see also Pusch *et al.*[31]).

BAY1436032 clears AML blasts *in vivo* and prolongs survival in PDX models of IDH1 mutant AML

Next, NSG mice were transplanted with primary AML cells from a patient with IDH1R132C mutant AML as described above for the PDX1 mouse model. Per condition 10 mice were treated with vehicle, 45 or 150 mg/kg body weight BAY1436032 once daily by oral gavage for 150 days starting 17 days after transplantation (Figure 3a–c). Serum R-2HG levels constantly increased in vehicle-treated mice reaching 62 μM at day 90 (Figure 3a). At 90 days serum levels of R-2HG were significantly lower in both the 45 and
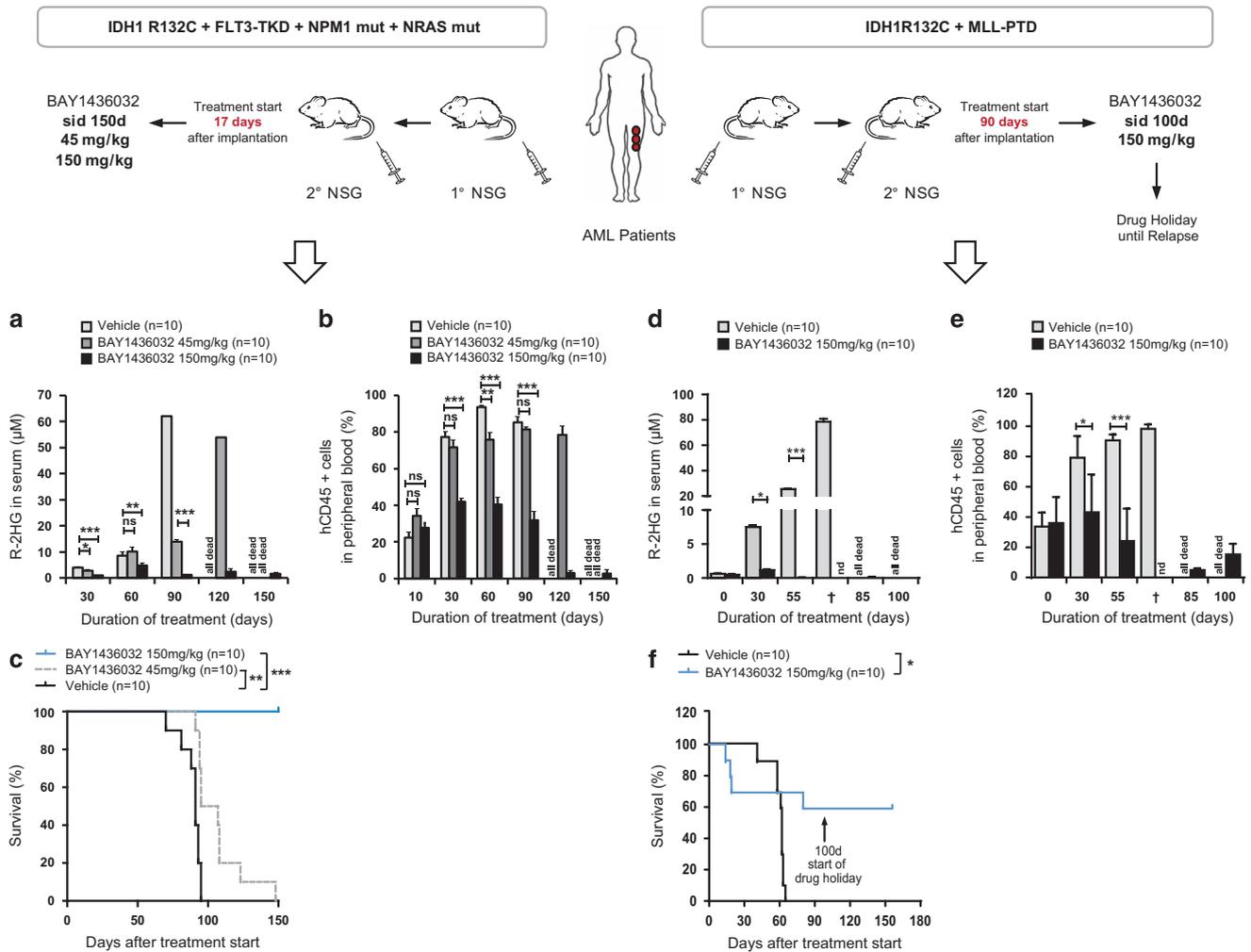
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Figure 3. BAY1436032 exerts anti-leukemic activity in IDH1R132C AML PDX mouse models *in vivo*. The experimental setup of two independent PDX models is given at the top. (**a**) R-2HG serum levels of PDX1-IDH1R132C mice treated with 45 or 150 mg/kg of BAY1436032 or vehicle once daily. Serum was pooled from 3–4 mice for R-2HG measurements by mass spectrometry (mean ± s.e.m., $n = 10$). (**b**) Percentage of hCD45$^+$ leukemic cells in peripheral blood of PDX1-IDH1R132C mice at different time points after treatment start with 45 or 150 mg/kg of BAY1436032 or vehicle (mean ± s.e.m., $n = 10$). (**c**) Survival of PDX1-IDH1R132C mice treated with 45 or 150 mg/kg of BAY1436032 or vehicle ($n = 10$). (**d**) R-2HG serum levels of PDX2-IDH1R132C mice treated with 150 mg/kg of BAY1436032 or vehicle once daily. Serum was pooled from all mice and R-2HG was measured in technical triplicates by enzymatic assay (mean ± s.d., $n = 10$). (**e**) Percentage of hCD45$^+$ leukemic cells in peripheral blood of PDX2-IDH1R132C mice at different time points after treatment start with 150 mg/kg of BAY1436032 or vehicle (mean ± s.d., $n = 10$). (**f**) Survival of PDX2-IDH1R132C mice treated with 150 mg/kg of BAY1436032 or vehicle ($n = 10$). Treatment was stopped at day 100. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not significant; ND, not determined; †, time of death.

150 mg/kg treatment groups (14 and 1 μM, respectively). Although serum R-2HG levels further increased in the 45 mg/kg group reaching 54 μM at day 120, R-2HG levels remained suppressed in the 150 mg/kg cohort until the end of the experiment at day 150. Accordingly, the percentage of human CD45$^+$ (hCD45$^+$) cells in the peripheral blood of the mice constantly increased in vehicle and 45 mg/kg BAY1436032 treated animals up to day 90, while it was significantly lower in the 150 mg/kg cohort and from day 30 onward declined to 2.8% at day 150 (Figure 3b). Similarly, white blood cell counts constantly increased in vehicle-treated mice and, at a lower rate, in animals receiving 45 mg/kg BAY1436032, while they remained constant in the 150 mg/kg cohort (Supplementary Figure 3A). Hemoglobin levels were slightly lower in the vehicle and 45 mg/kg groups as compared to the 150 mg/kg cohort at day 60 (Supplementary Figure 3B), while platelet counts were significantly reduced in vehicle and 45 mg/kg BAY1436032 treated mice compared to the 150 mg/kg cohort at day 60 (Supplementary Figure 3C). Strikingly, all mice receiving 150 mg/kg BAY1436032 survived with minimal hCD45$^+$ cell load in their

peripheral blood until the end of observation at day 150 after treatment start ($P < 0.001$), while vehicle-treated animals died from leukemia with a median survival of 91 days. Mice receiving 45 mg/kg BAY1436032 showed a small but significant increase in survival to a median of 101 days ($P = 0.002$; Figure 3c).

In an independent second PDX model (PDX2), NSG mice were transplanted with primary IDH1R132C AML cells, which harbored a *MLL*-PTD mutation as additional recurrent aberration. Treatment with vehicle or 150 mg/kg BAY1436032 once daily by oral gavage for 100 days was initiated in this model at day 90 after transplantation, thereby more closely resembling the situation of AML patients at diagnosis or relapse (Figure 3d–f). Similar to the first model, serum R-2HG levels constantly increased in vehicle-treated mice reaching 78.5 μM at death (Figure 3d). Again, R-2HG serum levels remained suppressed in the 150 mg/kg cohort until the end of BAY1436032 treatment at day 100. Accordingly, the percentage of hCD45$^+$ cells in the peripheral blood of the mice constantly increased in vehicle-treated animals until death, while it was significantly lower in the BAY1436032 cohort and from day
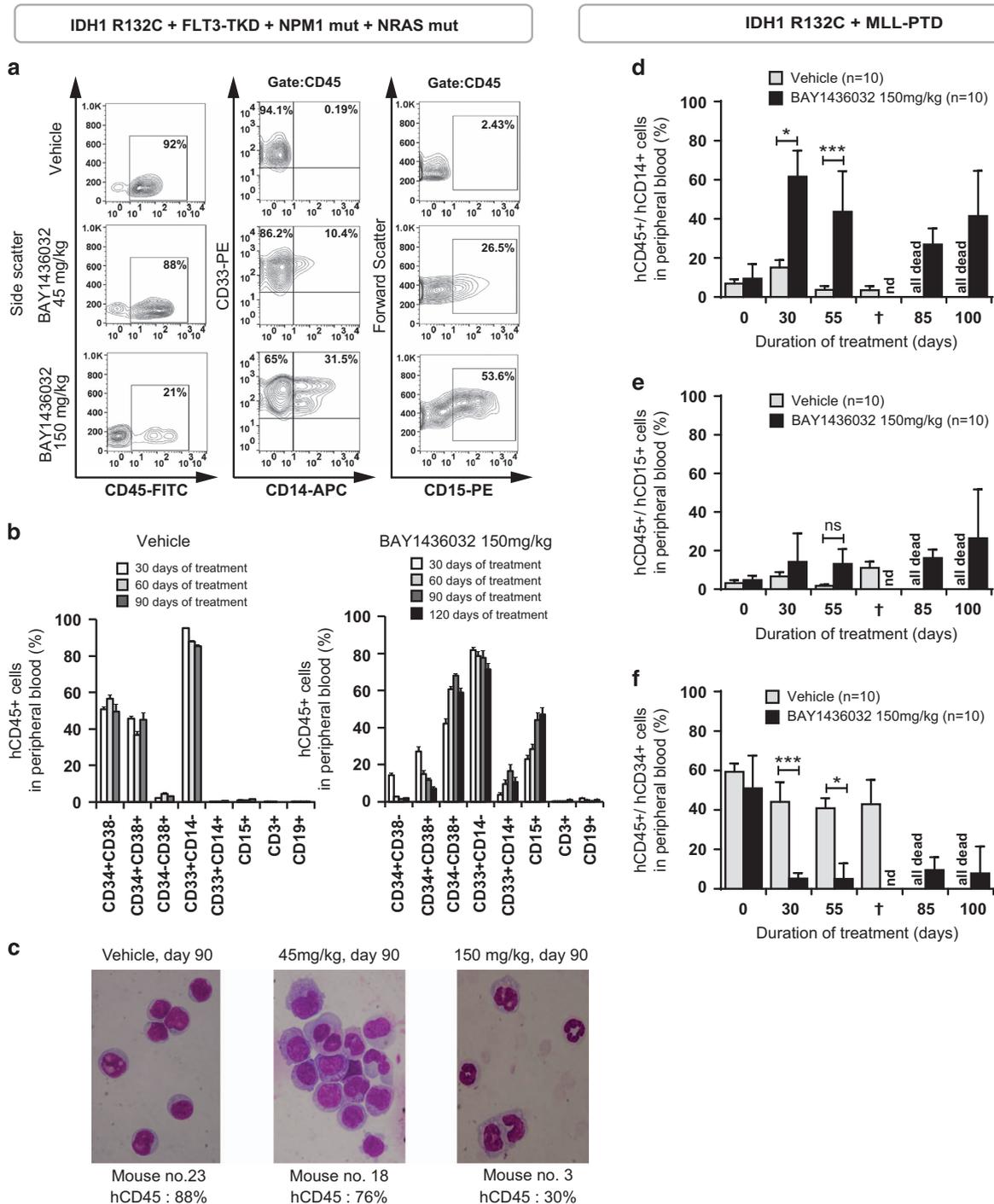
Figure 4. BAY1436032 induces myeloid differentiation of IDH1R132C mutant AML cells *in vivo*. (**a**) Representative FACS plots from peripheral blood cells of PDX1-IDH1R132C mice showing proportions of hCD45$^+$/CD14$^+$ and hCD45$^+$/CD15$^+$ cells at 90 days after treatment start with BAY1436032 or vehicle. (**b**) Immunophenotypes of hCD45$^+$ PDX1-IDH1R132C cells from mouse peripheral blood after treatment with vehicle (left) or 150 mg/kg BAY1436032 (right) at the indicated time points (mean ± s.e.m., $n = 10$). (**c**) Morphology of peripheral blood cells from PDX1-IDH1R132C mice treated with 45 or 150 mg/kg BAY1436032 or vehicle at the indicated time points (×1000 magnification). Percentages of hCD45$^+$/CD14$^+$ (**d**), hCD45$^+$/CD15$^+$ (**e**) and hCD45$^+$/CD34$^+$ (**f**) cells in the peripheral blood of PDX2-IDH1R132C mice treated with 150 mg/kg BAY1436032 or vehicle at the indicated time points after treatment start (mean ± s.d., $n = 10$). *$P < 0.05$; ***$P < 0.001$; NS, not significant; ND, not determined; †, time of death.

30 onward declined to 5.0% at day 85 and 15.3% at day 100 (Figure 3e). Total white blood cell counts increased in vehicle-treated mice until death, while they remained constantly low in the 150 mg/kg cohort (Supplementary Figure 3D). Both hemoglobin levels and platelet counts were reduced at death in the vehicle

group while they remained constant in the 150 mg/kg cohort until the end of BAY1436032 treatment at day 100 (Supplementary Figures 3E and F). Although again all vehicle-treated mice died from leukemia with a median survival of 62 days, 6 of 10 animals receiving 150 mg/kg BAY1436032 survived until day 100, when
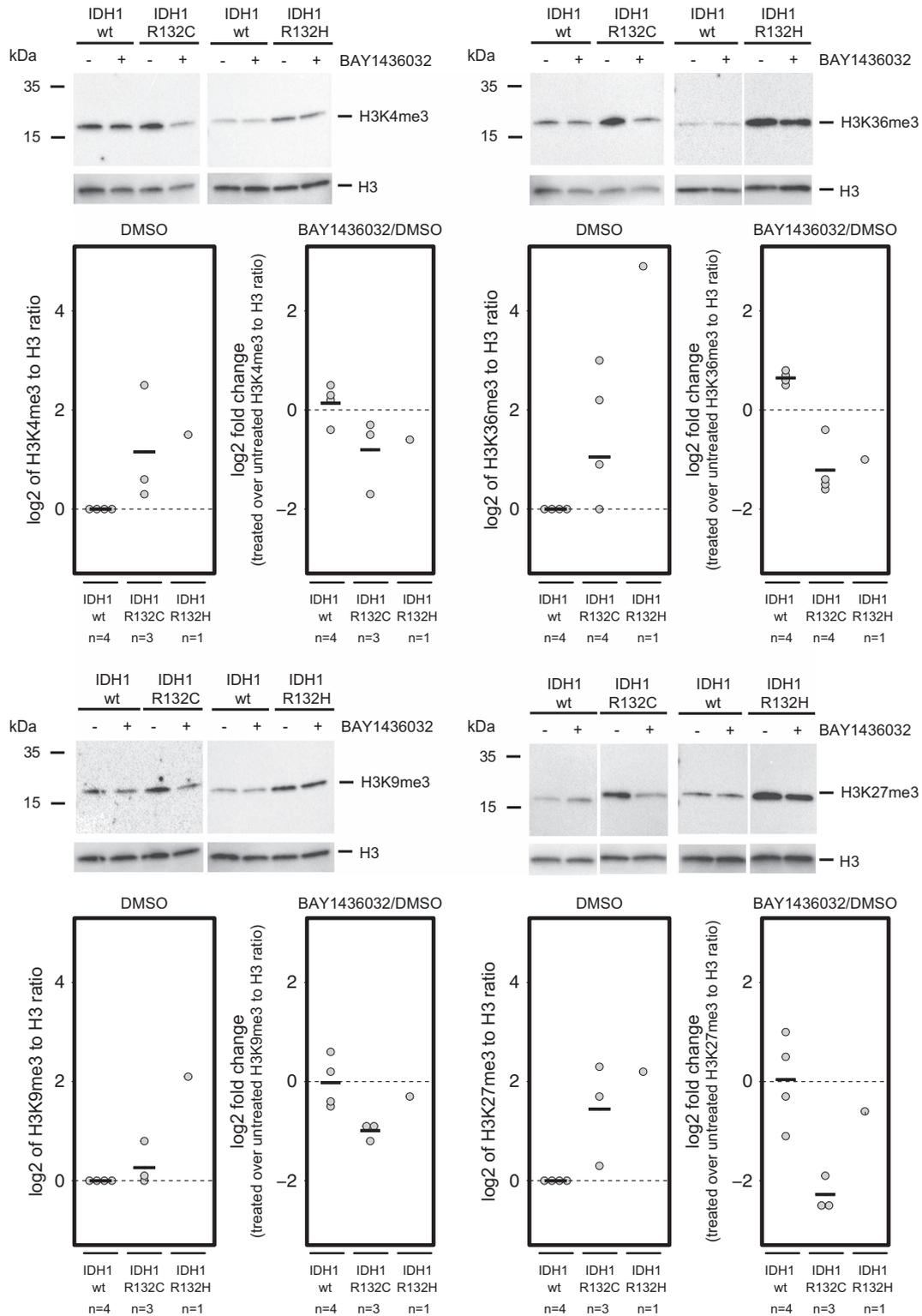
Figure 5. BAY1436032 impacts on histone methylation in patient-derived IDH1R132C and IDH1R132H mutant AML cells *ex vivo*. Western blots and quantification dot plots of histone H3 trimethylation levels at residues H3K4 (upper left), H3K36 (upper right), H3K9 (lower left) and H3K27 (lower right) in primary AML cells harboring wild-type (wt) IDH1 or IDH1R132C or IDH1R132H mutations. Cells were treated with 500 nM BAY1436032 or dimethyl sulfoxide (DMSO) as control for 14 days *ex vivo*. Log-transformed (base 2) ratios of the indicated histone modifications to total histone H3 levels in untreated IDH1 mutant cells compared to wild-type IDH1 cells (left panels) and log-transformed (base 2) fold changes of those ratios upon BAY1436032 treatment relative to DMSO control (right panels) were determined from the integrated intensity of the bands on the same western blot. The mean of logarithmized values is indicated for conditions with more than one measurement.

drug treatment was stopped (median survival not reached, $P = 0.014$; Figure 3f). Twenty-eight days after termination of BAY1436032 treatment the hCD45^{+} cell load in the peripheral blood of the surviving six mice was still minimal and remained low in 4 of 6 animals at day 56 (Supplementary Figure 4). In an independent third PDX model, NSG mice were transplanted with primary wild-type IDH1/IDH2, NPM1 (p.W288CfsTer12) mutant AML cells (PDX3). Treatment with vehicle or 150 mg/kg BAY1436032 once daily by oral gavage was initiated at day 15 after transplantation. BAY1436032 and vehicle-treated mice had similar percentages of hCD45^{+} cells, peripheral blood counts, spleen weight at death and survival, confirming the on-target activity of BAY1436032 (Supplementary Figure 5). We conclude that BAY1436032 exerts a striking and specific therapeutic effect at a dose of 150 mg/kg in PDX mouse models transplanted with primary human IDH1 mutant AML cells.

BAY1436032 induces myeloid differentiation in IDH1 mutant AML PDX models in vivo

To validate the induction of myelomonocytic differentiation by BAY1436032 in vivo the immunophenotype of hCD45^{+} cells from the peripheral blood of NSG mice transplanted with IDH1R132C mutant AML cells carrying additional FLT3, NPM1 and NRAS mutations (PDX1) was constantly monitored by flow cytometry. At day 90 after initiation of treatment, 94% of hCD45^{+} cells in vehicle-treated mice co-expressed CD33 but myelomonocytic maturation markers CD14 ($0.5 \pm 0.5\%$, $n = 2$) and CD15 ($1.7 \pm 0.5\%$, $n = 2$) were completely absent (Figure 4a). In contrast, mice treated with 150 mg/kg BAY1436032 harbored only few hCD45^{+} cells in their peripheral blood with a significant proportion of them co-expressing CD14 ($16.6 \pm 10.7\%$, $n = 2$) and CD15 ($44.2 \pm 11.6\%$, $n = 2$). Mice treated with 45 mg/kg BAY1436032 displayed intermediate levels of CD14/CD15 expression (Figure 4a). The immunophenotype of AML cells remained unchanged over time in vehicle-treated mice with high proportions of CD34^{+}CD38^{-} and CD34^{+}CD38^{+} progenitor cells (Figure 4b, left panel). Conversely, in mice treated with 150 mg/kg BAY1436032 the amount of CD34^{+} CD38^{-} and CD34^{+}CD38^{+} cells rapidly decreased while CD34^{-}CD38^{+}, CD33^{+}CD14^{+} and particularly CD15^{+} cells increased (Figure 4b, right panel). Morphologic evaluation confirmed blastic morphology of peripheral blood cells in vehicle-treated cells, while some differentiation towards myelocytes was noted in the 45 mg/kg group. Bands and neutrophils were the predominant morphology in mice treated with 150 mg/kg BAY1436032 (Figure 4c).

Similarly, in the independent second PDX model of NSG mice transplanted with primary IDH1R132C AML cells and an additional MLL-PTD mutation (PDX2), the immunophenotype of AML cells in vehicle-treated mice remained virtually unchanged over time until death with regard to constantly high levels of CD34 co-expression and a lack of CD14/CD15 upregulation (Figure 4d-f). In contrast, the amount of CD34 co-expressing AML cells rapidly decreased while CD14^{+} and CD15^{+} cells increased in the 150 mg/kg BAY1436032 cohort. After termination of BAY1436032 treatment the few remaining hCD45^{+} cells (Supplementary Figure 4A) in the peripheral blood of surviving mice progressively lost CD14 expression over time (Supplementary Figures 4B–D). Together, BAY1436032 induces myeloid differentiation at a dose of 150 mg/ kg in two independent PDX mouse models transplanted with primary human IDH1 mutant AML cells.

BAY1436032 depletes leukemic stem cells by induction of myeloid differentiation and inhibition of cell cycle progression

To assess the effect on leukemic stem cell (LSC) self-renewal we treated NSG mice transplanted with primary human IDH1R132C mutant AML cells (PDX1) with 150 mg/kg BAY1436032 or vehicle for 4 weeks. Subsequently a limiting dilution transplantation experiment with 3 NSG recipient mice per cell dose (200 000,

20 000, 2000, 200 and 20 hCD45^{+} cells per mouse) was performed. LSC frequency was determined using Poisson statistics. LSC frequency was 1 LSC in 84 hCD45^{+} cells in vehicle-treated mice, while the frequency was approximately 100-fold lower in BAY1436032 treated animals (1 LSC in 8683 hCD45^{+} cells, $P < 0.001$; Supplementary Figure 6A). Next, we performed gene expression profiling of hCD45^{+} cells from NSG mice four weeks after treatment with vehicle or 150 mg/kg BAY1436032. Expression of stemness-associated genes including SPARC, CD69 and DNMT3B was downregulated, while genes associated with myeloid differentiation and immune response were upregulated in BAY1436032 treated cells (GEO accession no. GSE83485; Supplementary Figures 6B and C and Supplementary Table 1). Gene set enrichment analysis revealed that one of the most highly enriched transcription factor gene sets upon treatment with BAY1436032 belonged to PU.1, while the top 20 gene sets for transcription factors that were negatively enriched in BAY1436032 treated cells were linked to the E2F family (Supplementary Figure 6D and Supplementary Table 2). Thus, BAY1436032 led to activation of the transcription factors PU.1, NFκB and AP1, while it inhibited E2F family transcription factors which promote progression from G_0/G_1 to S phase of the cell cycle (Supplementary Figure 6E). Accordingly, reduction of viable hCD45^{+} cells in peripheral blood and bone marrow of NSG mice upon treatment with BAY1436032 (Supplementary Figure 6F) was paralleled by increased frequencies of hCD45^{+} cells in G_0/G_1 and decreased frequencies in S phase of the cell cycle (Supplementary Figures 6G and 7). These data demonstrate that BAY1436032 not only induces differentiation but also inhibits cell cycle progression and self-renewal of LSCs in vivo.

BAY1436032 impacts on histone and DNA hypermethylation in primary human IDH1-mutated AML cells

The inhibition of histone demethylases by R-2HG results in a histone hypermethylation phenotype.[17–19] Accordingly, global histone H3 trimethylation levels at residues H3K4, H3K9, H3K27 and H3K36 were analyzed ex vivo by immunoblotting in primary human AML cells carrying either an IDH1R132C or an IDH1R132H mutation and compared to IDH1 wild-type AML cells. Levels were increased for all four histone methylation marks analyzed in cells with mutant IDH1 as compared to the corresponding global histone methylation levels in wild-type IDH1 AML cells (Figure 5). Upon treatment with 500 nM BAY1436032 for 14 days, histone tri-methylation levels decreased in both IDH1R132C and IDH1R132H mutant AML cells but not in IDH1 wild-type cells (Figure 5). These results show that BAY1436032 is specific for IDH1 mutant cells and can revert their histone hypermethylation phenotype.

In addition to histone hypermethylation, human AML cells with IDH1/IDH2 mutation show global DNA hypermethylation.[17,20] To test whether treatment with BAY1436032 alters DNA methylation, primary human AML cells carrying either an IDH1R132C or an IDH1R132H mutation were treated with 500 nM BAY1436032 for 14 days and subsequently subjected to analysis using the Illumina Infinium HumanMethylation450 platform. In this analysis BAY1436032 treated and vehicle-treated cells clustered together into clearly separable groups using Pearson correlation (Supplementary Figure 8A). In accordance with the gene expression profiling results, methylation of the PU.1 promoter decreased while E2F1 promoter methylation increased upon treatment with BAY1436032 (Supplementary Figures 8B–E). Together, these data demonstrate that BAY1436032 impacts on DNA methylation and suggests that the compound differentially alters methylation patterns to upregulate PU.1 and downregulate E2F gene expression.

DISCUSSION

Here we show that BAY1436032 selectively inhibits mutant IDH1 leading to potent suppression of R-2HG production, inhibition of proliferation, and induction of myeloid differentiation in primary IDH1 mutant AML cells *in vitro*. In two independent PDX mouse models of primary IDH1 mutant AML BAY1436032 prolongs survival by causing blast clearance and myeloid differentiation as well as inhibition of stem cell self-renewal.

Several inhibitors of mutant IDH isoforms that block R-2HG production *in vitro* and *in vivo* have recently been described. The first potent and specific IDH1 inhibitors reported were AGI-5198[refs 32,33] and, subsequently, ML309.[34,35] These compounds specifically inhibited IDH1R132H and were shown to inhibit 2HG production and induce differentiation in glioma cells. Subsequently, Zheng and coworkers reported two potent inhibitors of IDH1R132H and IDH1R132C with greater than 60-fold selectivity relative to wild-type IDH1.[36] In a recent study the pan-IDH1 inhibitors GSK321 and GSK864 decreased R-2HG production in several different IDH1 mutant primary AML cells *ex vivo*.[29] *In vivo*, GSK864 reduced leukemic blast counts, but improved survival has not been reported for any of these compounds yet. AG-120, a selective, potent inhibitor of mutant IDH1, is currently in clinical development for the treatment of cancers with IDH1 mutations. Preliminary data show that it causes myeloid differentiation and induces remissions in up to 36% of IDH1 mutant AML patients.[30] However, the allele burden of mutant IDH1 remained high in several responding patients, suggesting that LSCs are not depleted.[30] Here we developed and evaluated the orally bioavailable pan-IDH1 inhibitor BAY1436032 for its anti-leukemic effect in PDX mouse models of primary human IDH1 mutant AML.

BAY1436032 specifically inhibited the mutant IDH1 enzyme and decreased intracellular R-2HG levels in both mouse hematopoietic cells transfected with mutant versions of IDH1 and primary AML cells with a wide range of different IDH1 mutations. Consequently, the compound enhanced myeloid differentiation both *in vitro* and *in vivo* along with decreased colony formation *in vitro* and reduced cell cycle progression and blast clearance *in vivo*. In tissue culture, BAY1436032 led to upregulation of the myelomonocytic markers CD14 and CD15, which was paralleled by morphologic differentiation. Similar observations in AML have been reported by others.[29] The differentiation block induced by mutant IDH1 might be due to histone hypermethylation with pronounced accumulation of trimethylated histones as reported previously.[17–20] We found here that treatment with BAY1436032 led to reduced levels of H3K4me3, H3K9me3, H3K27me3 and H3K36me3 marks in primary IDH1 mutant AML, analogous to what has been described for H3K9me2 by GSK321 in IDH1R132C expressing HT-1080 cells.[29]

In accordance with myeloid differentiation and reduced proliferation, gene expression profiling revealed that treatment of PDX mice with BAY1436032 led to reduced expression of stemness-associated genes and inhibition of E2F transcription factors. In contrast, the activity of myeloid transcription factors like PU.1 was upregulated in hCD45+ AML cells. A similar cell cycle arrest in G1 phase has been reported for IDH1 mutant AML cells treated with GSK321.[29] Although not significant, concordant demethylation of the PU.1 gene promoter and increased methylation of the E2F promoter was found by DNA methylation analysis. However, no significant difference in global DNA methylation was found, a result that is in line with the previous data from glioma xenotransplants treated with AGI-5198[ref. 33] or BAY1436032.[31]

Three other IDH1 inhibitors—AGI-5198, GSK321 and AG-120—have been shown to induce differentiation of IDH1 mutant glioma or AML cells.[29,30,33] Moreover, AG-120 was recently reported to even induce differentiation syndrome concurrently with clinical response in patients with IDH1 mutant AML.[37] Nevertheless, allele burden of mutant IDH1 remained high in these patients,[30]

suggesting that AG-120 does not deplete LSCs. In contrast, BAY1436032 inhibits stem cell self-renewal as shown by low stem cell numbers in BAY1436032 treated mice upon secondary transplantation.

Myelomonocytic differentiation upon drug treatment occurred in both models and was similar to the *ex vivo* results. Reminiscent of clinical differentiation syndrome in AML patients treated with AG-120[37] we found an increased early mortality in our second PDX mouse model with primary IDH1R132C AML cells and an additional *MLL*-PTD mutation, in which mice suffered from a high leukemic burden when treatment was established at three months after transplantation. In contrast, in PDX1-IDH1R132C mice, which received BAY1436032 already at day 17 after transplantation, no early mortality occurred.

Once a day oral administration of 150 mg/kg BAY1436032 led to leukemic blast clearance and significantly prolonged survival in two independent PDX models of primary human IDH1 mutant AML. R-2HG production in PDX mice was rapidly inhibited after administration of BAY1436032. With 45 mg/kg BAY1436032 the reduction of R-2HG levels was first noted 7 h after treatment start. With 90 and 150 mg/kg, significant reduction of R-2HG levels occurred already 3 h after the first dose. While 45 mg/kg BAY1436032 only partially suppressed R-2HG production in long-term dosing experiments, it was insufficient to fully prevent expansion of leukemic cells and only moderately prolonged survival of PDX mice. In contrast, 150 mg/kg BAY1436032 led to almost complete suppression of R-2HG production, profound reduction of leukemic burden and significantly prolonged survival. Of note, even two months after termination of BAY1436032 treatment leukemic burden in the peripheral blood of two thirds of the remaining mice was still minimal, further arguing in favor of a significant depletion of the LSC pool.

In summary, the novel pan-mutant IDH1 inhibitor BAY1436032 is active against all IDH1R132 mutations and, when orally applied at a dose of 150 mg/kg, shows strong anti-leukemic activity in two independent AML PDX mouse models. On the basis of the present work on BAY1436032 a clinical phase one trial enrolling patients with IDH1R132 mutant AML is currently being initiated.

AUTHOR CONTRIBUTIONS

AK, MH, AC and LH conceived and designed the study; AC, LH, SP, LK, RG, DS, SK, TB, EAS, AS, AB, RG, MMA-C, FT, RG, AG, ADH, AD, KR, MH and AK collected, analyzed, and assembled the data; OP, KZ, LT, RN, AH, HR, HH-S and MB provided critical reagents; AK and MH wrote the manuscript; and all authors reviewed the data, and edited and approved the final version of the manuscript.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)