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Synergistic activity of IDH1 inhibitor BAY1436032 with azacitidine in IDH1 mutant acute myeloid leukemia

Anuhar Chaturvedi,[1*] Charu Gupta,[1] Razif Gabdoulline,[1] Nora M. Borchert, [1] Ramya Goparaju,[1] Stefan Kaulfuss,[2] Kerstin Görlich,[1] Renate Schottmann,[1] Basem Othman,[1] Julia Welzenbach,[3] Olaf Panknin,[2] Markus Wagner,[2] Robert Geffers,[4] Arnold Ganser,[1] Felicitas Thol,[1] Michael Jeffers,[5] Andrea Haegebarth,[2] and Michael Heuser[1*]

[1]Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School, Hannover, Germany

[2]Bayer AG, Berlin, Germany

[3]Institute of Human Genetics, University of Bonn, School of Medicine & University Hospital Bonn, Bonn, Germany

[4]Genome Analytics Research Group, Helmholtz Centre for Infection Research, Braunschweig, Germany

[5]Bayer AG, Whippany, NJ, USA

Additional footnotes: * Corresponding authors

Corresponding Authors:

Michael Heuser, MD and Anuhar Chaturvedi, PhD

Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School, Carl-Neuberg Strasse 1, 30625 Hannover, Germany.

Phone: +49-511-532-3720

Fax: +49-511-532-3611

E-mail: heuser.michael@mh-hannover.de, chaturvedi.anuhar@mh-hannover.de

Running Title: Synergy between IDH1 inhibitor and azacitidine

Abstract

Mutant IDH1 (mIDH1) inhibitors have shown single-agent activity in relapsed/refractory AML, though most patients eventually relapse. We evaluated the efficacy and molecular mechanism of the combination treatment with azacitidine, which is currently the standard of care in older AML patients, and mIDH1 inhibitor BAY1436032. Both compounds were evaluated in vivo as single agents and in combination with sequential (azacitidine, followed by BAY1436032) or simultaneous application in two human IDH1 mutated AML xenograft models. Combination treatment significantly prolonged survival compared to single agent or control treatment (P<.005). The sequential combination treatment depleted leukemia stem cells (LSC) by 470-fold. Interestingly, the simultaneous combination treatment depleted LSCs by 33,150-fold compared to control mice. This strong synergy is mediated through inhibition of MAPK/ERK and Rb/E2F signaling. Our data strongly argues for the concurrent application of mIDH1 inhibitors and azacitidine and predicts improved outcome of this regimen in IDH1 mutated AML patients.

Introduction

In acute myeloid leukemia (AML) patients, mutated *isocitrate dehydrogenase 1* (*IDH1)* is found in about 6-10% of patients [1, 2] at amino acid arginine 132 in the active site where isocitrate and NADPH bind.[3] Whereas the enzymatic function of mutant IDH to convert isocitrate to α-ketoglutarate is impaired, it gains a neomorphic function to convert α-ketoglutarate to R-2-hydroxyglutarate (R-2HG), which induces histone- and DNA hypermethylation through inhibition of demethylation, and leads to a block in cellular differentiation thus promoting tumorigenesis.[3-6] IDH1 mutant-specific inhibitors have emerged as a potent tool for the treatment of IDH1 mutant AML. A recent report on the first clinical IDH1 inhibitor ivosidenib as a single agent in *IDH1*-mutated relapsed or refractory AML showed an overall response rate of 41.6% and a complete

remission rate of 21.6% with a median duration of response of 8.2 months.[7] While these results are promising in this difficult to treat patient setting, they also suggest that mIDH1 inhibitors should be combined with other agents to improve efficacy.

A possible mechanism of synergy in IDH1 mutant AML is the reversal of DNA methylation by hypomethylating agents. 5-azacitidine (AZA) is a hypomethylating agent and can activate key epigenetically silenced pathways in AML cells, leading to an arrest of AML cell proliferation.[8] The AZA-AML-001 study showed that azacitidine has clear activity in AML as monotherapy in patients with unfavorable cytogenetics.[9] Therefore, a successful reversal of IDH1 mutant induced DNA hypermethylation in AML cells represents a window of opportunity to enhance the efficacy of mIDH1 inhibitors.

We have previously shown that BAY1436032 is a highly effective oral pan-mutant IDH1 inhibitor, which has strong anti-leukemic activity in patient-derived xenograft (PDX) models of IDH1 mutant leukemia *in vivo* by inducing differentiation of leukemic blasts as well as inhibition of leukemia stem cell proliferation and self-renewal [10]. BAY1436032 is currently evaluated in phase 1 clinical trials in AML and glioma patients (NCT03127735 and NCT02746081).

In this study, we evaluated the therapeutic efficacy and elucidated the mechanism of action of the combined treatment of azacitidine and BAY1436032 in IDH1 mutant AML *in vitro* as well as *in vivo* in two independent patient-derived xenograft models of IDH1 mutant AML. From these experiments, we conclude that the simultaneous combination of a mIDH inhibitor with a hypomethylating agent synergistically inhibits LSCs through suppression of MAP kinase and RB/E2F signaling, which are involved in cell survival and proliferation.

Methods

Combination index

Drug synergy was evaluated using a combination index (CI) equation based on the multiple drug-effect equation of Chou-Talalay.[11, 12] Colony-forming cell units were assayed in methylcellulose after treatment with varying doses of either azacitidine (62.5, 125, 250, 500, 1000 or 2000 nm), BAY 1436032 (6.25, 12.5, 25, 50, 100 or 200 nm) or a fixed ratio of the combination of azacitidine/BAY1436032 (62.5/6.25, 125/12.5, 250/25, 500/50, 1000/100 or 2000/200 nm). The analysis was performed with CompuSyn software (ComboSyn Inc., Paramus, USA).

Patient Samples

Diagnostic bone marrow or peripheral blood collected from AML patients at Hannover Medical School were analyzed for mutations in *IDH1* and *IDH2* by Sanger sequencing. Details of the type of IDH mutation are described in the figure legends. Mononuclear cells were isolated by ficoll density centrifugation, washed with PBS, and red blood cells were lysed using an RBC lysis buffer (BD Pharm Lyse, BD Biosciences, Heidelberg, Germany). The bone marrow samples for the development of the PDX models were collected prior to the start of AML treatment. Written informed consent was obtained according to the Declaration of Helsinki, and the study was approved by the institutional review board of Hannover Medical School, Hannover, Germany.

Transplantation and treatment

6-8 weeks old female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from Hannover Medical School, Germany and kept in pathogen free conditions at the central animal

laboratory of Hannover Medical School. Experimental procedures were approved by governmental authorities of the Lower Saxony, Germany, and supervised by local animal welfare officials. The IDH1mut AML PDX model was developed as described.[10] One million patient-derived AML cells (hCD45+) were collected from bone marrow and spleen of leukemic mice and were injected intravenously in the tail vein of sublethally (3 Gy) irradiated NSG mice. Neither randomization, nor blinding was used since all animal experiments were performed with a homogeneous strain, comparable age, and similar variance of the mice. Treatment was started 28 days after transplantation. The control groups were treated with either vehicle, BAY1436032 150 mg/kg once daily p.o. continuously, or azacitidine 1 mg/kg once daily s.c. days 1-5, repeated once after 28 days. The test groups were treated with BAY1436032 and azacitidine in the doses mentioned above either starting both drugs on day 1 (simultaneous group) or starting azacitidine on day 1 but BAY1436032 on day 6 (sequential group). In the second cycle in the sequential treatment group, BAY1436032 was stopped during azacitidine treatment to better model the biologic and therapeutic effects of the synergy. The treatment was stopped at day 84 or day 96. The proportion of leukemic cells in peripheral blood of mice was measured with the human-specific CD45 antibody every four weeks by tail vein bleeds and FACS analysis. [13] Blood counts were performed using an ABC Vet Automated Blood counter (Scil animal care company GmbH, Viernheim, Germany). All animal experiments were started with 10 animals, however, the animals which died before the start of treatment due to engraftment failure, during injections or during bleeding were excluded from the study.

Statistical analysis

Two tailed unpaired comparisons were performed by Mann-Whitney test in Graph pad prism 8.2.1 (GraphPad Software, La Jolla, CA). Comparison of survival curves were performed using the log-rank test. Statistical analyses were performed with Microsoft Excel 2016 (Microsoft,

Munich, Germany) or GraphPad Prism 8.2.1 (GraphPad Software, La Jolla, CA). Graphical representation was prepared using Adobe Illustrator CS6 (Adobe systems GmbH, Munich, Germany). The size of the animal cohorts was based on our previous study. [5, 6, 10] All in vitro experiments were performed at least 3 times and all attempts of replication were successful.

Results

Mutant IDH1 inhibitor BAY1436032 and azacitidine synergize to inhibit human IDH1 mutant AML cells ex vivo.

To test the effect of the combination of BAY1436032 and azacitidine on survival and proliferation of primary human AML cells, IDH1 wildtype (n=6) and IDH1 mutant (n=6) cells from AML patients were seeded in semi-solid medium supplemented without or with BAY1436032 in combination with varying concentrations of azacitidine. BAY1436032 reduced colony formation specifically in human IDH1 mutated AML cells with an IC_{50} of 100 nM, while IDH1 wildtype cells were not affected (Supplementary Figure S1A). Azacitidine alone induced a 20% reduction in colony formation in both IDH1wt and IDH1mut AML patient cells at 100 nM (Supplementary Figure S1B), while the combination of BAY1436032 with azacitidine reduced colony formation in a dose-dependent manner in IDH1 mutated cells with reduction of colonies by 80% in IDH1 mutated AML but only by 20% in IDH1 wildtype AML cells at 100 nM azacitidine, suggesting improved efficacy of an IDH1 inhibitor in combination with azacitidine (Figure 1A, normalized to cells treated with BAY1436032 at 100 nM). To test the effect on cell cycle progression and apoptosis, IDH1 mutant and IDH1 wildtype AML cells were cultured in suspension medium and treated with vehicle, 100 nM BAY1436032 or 100 nM azacitidine as single agents or in combination. The proportion of cells in S phase of the cell cycle was strongly decreased by the combination treatment compared with either monotherapy or vehicle in IDH1 mutant AML cells, however, remained largely unchanged in IDH1 wildtype cells (Figure 1B and 1C). 'No difference

was observed in the proportion of G0/G1 cells between the combination and monotherapy groups, while the subG1 population was increased in the combination treatment group indicating increased cell death. The percentage of Annexin V+ cells did not differ between any of the treatment groups in IDH1wt or IDH1mut AML cells (Supplementary Figure S2). To further investigate the synergy between azacitidine and BAY1436032, we treated IDH1mut AML cells from six patients with serial dilutions of azacitidine and BAY1436032, either alone or in combination and performed colony forming unit assays. The combination of azacitidine with BAY14360932 significantly decreased the colony formation of cells treated with both agents (Figure 1D). To determine whether the combined effects of azacitidine and BAY1436032 are additive or synergistic, we performed an isobologram analysis from an average of 6 patients. The combination of azacitidine with BAY1436032 proved highly synergistic with a combination index (CI) of < 0.68 at an effective dose (ED) of 95, a CI of < 0.65 at ED 75, and a CI of < 0.63 at ED 50, respectively (Figure 1E; a CI of 1 reflects an additive effect, a CI < 1 a synergistic effect and a CI > 1 indicates antagonism). This data suggest that BAY1436032 in combination with azacitidine more effectively inhibits proliferation of primary IDH1 mutated AML cells ex vivo than single agent treatment.

BAY1436032 synergizes with azacitidine to exert potent anti-leukemic activity in the patient-derived IDH1 mutant AML xenograft models *in vivo*.

We have previously developed and characterized an IDH1 mutant PDX mouse model (AML-PDX1) using primary AML cells from a patient harboring IDH1R132C, FLT3-TKD (p.D835del), an atypical NPM1 (p.S254LfsTer4), and a NRAS (p.Q61R) mutations. [10] After 28 days of transplantation, having confirmed engraftment between 2% to 5% in peripheral blood, IDH1 mutant PDX mice were treated with either vehicle, BAY1436032, two cycles of azacitidine as monotherapy or in sequential or simultaneous combination of both drugs for a total duration of 84 days (Figure 2A). We evaluated the simultaneous and sequential combination treatment to

derive mechanistic insight into the combination treatment and to instruct the clinical use of this combination, as mutation analysis usually takes some time and leaves a time window in which treatment with azacitidine may be started before the mutation status is known, followed by sequential treatment with a mIDH1 inhibitor.

While the engraftment of human leukemic cells increased in vehicle and azacitidine treated mice at week 8 after the start of treatment, the percentage of leukemic cells decreased in BAY1436032 treated mice as well as in the groups receiving the sequential and simultaneous combination treatment (Figure 2B). However, after the stop of treatment at week 12 the percentage of leukemic cells increased after week 16 in the treatment groups receiving BAY1436032 or the sequential combination of BAY1436032 and azacitidine (Figure 2B). Interestingly, the percentage of leukemic cells in mice treated with the simultaneous combination of BAY1436032 and azacitidine showed a delayed increase of blasts and slower kinetics (Figure 2B) and 2 of 6 mice from this cohort remained negative in peripheral blood until the end of the study at 36 weeks (Figure 2C). The mean IDH1 mutant allele fraction in peripheral blood at 8 weeks after the stop of the treatment was 48.8% in vehicle, 40.56% in the azacitidine, 46.43% in the BAY1436032, and 43.2% in the sequential treatment cohorts. However, median IDH1 mutant allele burden was 10.89% in the simultaneous treatment cohort, and below the detection limit in 3/5 analysed mice (Supplementary Figure S3A). While the WBC counts constantly increased and platelet counts, as well as hemoglobin, decreased in mice receiving vehicle, azacitidine or BAY1436032 monotherapy or the sequential combination of BAY1436032 and azacitidine, all mice treated simultaneously had normal blood counts until week 36 (Figure 2D, 2E, and Supplementary Figure S3B). To compare the effects of the combination therapy with single agents on the induction of myelomonocytic differentiation the expression of CD14 and CD15 in human CD45+ cells was monitored. At day 30 after initiation of treatment there was no significant difference in the expression of myelomonocytic maturation markers CD14 and CD15 in the vehicle (CD14 0.05±0.05; CD15 0.86±0.3) and azacitidine

treated groups (CD14 0; CD15 1.39±0.36). In contrast, mice treated with BAY1436032 had a significantly higher proportion of cells expressing CD14 (4.21±0.62) and CD15 (7.67±0.68). Mice treated in parallel with the combination of BAY1436032 and azacitidine had additive effects on the expression of these markers (CD14 7.44±1.27; CD15 11±0.79) (Supplementary Figure S4). While azacitidine treated mice survived longer with a median survival of 188 days compared to vehicle-treated mice with a median survival of 139 days, BAY1436032 treated mice had significantly longer latency with a median survival of 220 days. However, mice treated sequentially with the combination of BAY1436032 and azacitidine survived longer than with BAY1436032 monotherapy with a median survival of 299 days. Strikingly, 5/6 mice treated simultaneously with BAY1436032 and azacitidine survived until the end of the study at 300 days and the median survival was not reached (Figure 2F).

In an independent, second PDX model (AML-PDX2), which harbored *IDH1* p.R132H*, DNMT3A* p.R882H, *PTPN11* p.A72T, and *NPM1* p.T288CfsTer12 mutations (Supplementary Figure S5A), the percentage of human CD45+ cells increased in vehicle, azacitidine, BAY1436032 and sequential combination treatment groups albeit with different kinetics (Supplementary Figure S5B). However, even after the stop of treatment at week 12, the percentage of leukemic cells in mice treated with the simultaneous combination of BAY1436032 and azacitidine remained low and 2 of 6 mice from this cohort had less than 10% human CD45+ cells in peripheral blood until the end of the study at 28 weeks (Supplementary Figure S5C). In contrast to the other groups, all mice treated simultaneously had normal blood counts until week 16 after the start of treatment (Supplementary Figure S5D-S5F). At day 90 after initiation of treatment mice treated in simultaneously with the combination of BAY1436032 and azacitidine had significantly higher expression of CD15 than mice treated with BAY1436032 alone or with the sequential combination of BAY1436032 and azacitidine (Supplementary Figure S5G). The mice treated simultaneously with BAY1436032 and azacitidine survived significantly longer with a median

survival of 250 days than sequentially treated mice with a median median survival of 186 days (Supplementary Figure S5G).

In summary, the simultaneous combination of BAY1436032 and azacitidine is highly effective in inhibiting IDH1 mutated human leukemia in vivo, suggesting a specific dependency on the simultaneous presence of both drugs *in vivo*.

Combination treatment with BAY1436032 and azacitidine strongly depletes leukemia stem cells *in vivo* through inhibition of MAP-kinase signaling and activation of myeloid differentiation.

To assess the effect of simultaneous and sequential treatment with BAY1436032 and azacitidine on leukemia stem cell self-renewal we performed a limiting dilution transplantation experiment with IDH1 mutant AML cells. NSG mice transplanted with primary human IDH1R132C mutant AML cells were treated when leukemias were fully established (70-80% human AML cells in peripheral blood) with either vehicle, azacitidine, BAY1436032, or the sequential or simultaneous combination of BAY1436032 and azacitidine for four weeks (Supplementary Figure S6A). Subsequently, we harvested bone marrow cells from these treatment groups and transplanted defined numbers of human AML cells in recipient mice at limiting dilution. Cell numbers of 2,000,000, 200,000, 20,000, 2,000, 200, and 20 human CD45+ AML cells were injected into three NSG recipient mice per cell dose for each treatment group. After 8 weeks the mice were bled and scored positive if more than 0.1% human CD45+ cells were present in peripheral blood (Figure 3A). The LSC frequency was 1 in 73 in vehicle-treated mice, 4.1-fold lower in azacitidine treated mice (1 in 304), and 117-fold lower in BAY1436032 treated mice (1 in 8580). The sequential combination treatment of BAY1436032 and azacitidine decreased the stem cell frequency by 470-fold (1 in 34,300) compared to control treated mice and by 4-fold compared to BAY1436032 monotherapy. However, in mice treated simultaneously

with BAY1436032 and azacitidine, the leukemia stem cell frequency was 33,150-fold lower compared to control treated mice, 282-fold lower compared to BAY1436032 treated mice, and 70-fold lower compared to mice treated sequentially with BAY1436032 and azacitidine (1 in 2,420,000, Figure 3A and Supplementary Figure 6B).

Next, we performed gene expression profiling of hCD45+ cells from NSG mice treated with vehicle, azacitidine, BAY1436032 or simultaneous treatment with BAY1436032 and azacitidine for 4 weeks. In unsupervised hierarchical clustering, azacitidine and vehicle-treated groups clustered together and separated from BAY1436032 and BAY1436032 + azacitidine treated groups. However, many genes that were overexpressed in BAY1436032 treated cells were suppressed in the combination group (Figure 3B). Principal component analysis showed that BAY1436032 + azacitidine treated cells separated well from the control or single-agent treated cells (Figure 3C). Interestingly, 11 out of 17 genes from the LSC17 gene signature were downregulated by the combination treatment, supporting the inhibitory effect of the combination therapy on LSCs (Supplementary Figure S7).[14] Gene set enrichment analysis revealed that the suppressed transcription factors in the combination treatment group were linked to MAP kinase and Retinoblastoma/E2F (Rb/E2F) signaling (Figure 3D and Supplementary Tables S1 to S3). We also performed epigenome wide asscociation studies by DNA methylation arrays and compared them with the transcriptome obtained by RNA sequencing (RNASeq) in IDH1 R132C cells isolated from AML-PDX1 mice and treated in vitro with either DMSO, 500 nM azacitidine (day 1 to 4), 50 nM of BAY1436032 (day 1 to 5), and sequential or simultaneous treatments with BAY1436032 and azacitidine. Principal component analysis showed that sequentially treated cells separated well from simultaneously treated cells (Supplementary Figure S8 A-B). The simultaneous treatment induced strong synergistic gene expression (S-value >0), while the gene expression was mostly antagonistic in the sequential treatment group (S-value <0; Supplementary Figure S8C). The simultaneous treatment overall led to increased methylation compared to sequential treatment (Supplementary Figure S8D). Gene set enrichment analysis

revealed that the suppressed transcription factors in the simultaneous treatment group were linked to MAP kinase signaling (RAS/RAF), while transcription factors enriched in single treatments were linked to Rb/E2F signaling (Supplementary Figure 8E). In concordance with gene expression, an epigenome-wide association showed increased methylation of transcription factors involved in MAP kinase (RAS/RAF) signaling upon simultaneous treatment, while it showed decreased methylation of gene sets involved in RB/E2F signaling upon single agent treatment (Supplementary Figure 8F). Gene expression analysis by quantitative RT-PCR validated an additive suppression of MAP kinase (*ELK1, ETS1* and *CCND1*) and E2F signaling (*E2F1, CCNA2* and *CCNE1*) by the combination treatment, which are involved in cell survival and proliferation. Myeloid differentiation genes (*PU.1, CEBPA* and *GABPA*) were upregulated by the combination of BAY1436032 and azacitidine, which might be due to the higher proportion of differentiated human CD45+ cells and may not be a direct effect of the drugs. (Figure 3E and Supplementary Figure S9A-9C). We further validated our findings at protein level in HT1080, a fibrosarcoma cell line with an endogenous heterozygous *IDH1R132C* mutation. The phosphorylation of ERK1/2 and expression of its downstream target proteins ELK1 and CYCLIN D1 was decreased in cells simultaneously treated with BAY1436032 and azacitidine. Importantly, the phosphorylation of retinoblastoma (pRB), which is necessary for E2F mediated transcription of cell cycle transition genes, was strongly decreased in cells treated with the combination of BAY1436032 and azacitidine, while E2F1 protein levels were not affected (Figure 3F). We next evaluated if targeting the MEK pathway or cyclin dependent kinases alter the proliferation of IDH1 mutated AML cells using using the MEK1/2 inhibitor trametinib and the CDK4/6 inhibitor abemaciclib. Trametinib 4-fold more potently inhibited colony-forming potential in IDH1 mutated compared to IDH1 wildtype patients (Fig. 3G). Similarly, the CDK4/6 inhibitor abemaciclib 3-fold more potently inhibited the colony-forming potential in IDH1 mutated compared to IDH1 wildtype patients (Fig. 3F). This data suggests synergy between BAY1436032 and azacitidine in induction of differentiation through transcriptional activation of

myeloid differentiation genes and inhibition of proliferation and self-renewal through inhibition of MAP-kinase and RB/E2F signaling.

Discussion

Here we provide preclinical evidence that the simultaneous combination of a mIDH1 inhibitor with a hypomethylating agent significantly inhibits LSCs in a synergistic manner. While the phase 1 dose-escalation study of mIDH1 inhibitor ivosidenib has shown encouraging results, 79% of IDH1 mutant AML patients who had a complete remission or complete remission with partial hematologic recovery had detectable allele burden of mutant IDH1, suggesting that the IDH1 mutant clone was not eradicated.[7] It was shown previously that BAY1436032 has antileukemic activity, induces differentiation and depletes LSCs 100-fold during a 4 week treatment.[10] However, BAY1436032 neither has anti-leukemic activity nor induces myeloid differentiation in an *IDH1* wildtype AML PDX model in vivo.[10] Azacitidine also induces differentiation and reduces self-renewal [15, 16] letting us hypothesize that the combination of a mIDH1 inhibitor and azacitidine may act synergistically. Our data suggest that the combination of BAY1436032 and azacitidine has additive effects on differentiation, while it synergistically reduces LSCs by 33,150-fold.

It was shown previously that mutant IDH1 activates MAPK/ERK signaling in leukemia and glioma cells.[5, 17] Therefore, we expected that a mIDH1 inhibitor would be able to reduce - MAPK/ERK signaling. Data from pancreatic cancer cells suggested that treatment with azacitidine also inhibits MAPK/ERK signaling by promoter hypomethylation and induction of expression of DUSP6, which dephosphorylates members of the MAPK superfamily.[18, 19] However, the effect of azacitidine on MAPK signaling in leukemia patients is not known. We found that inhibition of the MAP kinase pathway is not a direct consequence of DNA

hypomethylation of MAP kinase genes, but rather a transcriptional or posttranscriptional effect on genes that regulate the MAP kinase pathway.

We evaluated the consequences of MAPK/ERK inhibition and found that reduced phosphorylation of ERK1/2 leads to suppression of cyclin D1, which then is unavailable to bind CDK4. CDK4 in turn cannot phosphorylate Rb and consequently phosphorylation of Rb was strongly inhibited by the combined treatment of BAY1436032 and azacitidine, providing a mechanistic basis for reduced proliferation and self-renewal of AML cells (Figure 4). Phosphorylation of Serine 795 of Rb is required for the dissociation of E2F1 from Rb, so that it can bind to DNA and induce transcription of genes involved in the G1 to S transition.[20] Similarly, the phosphorylation of Serine 807 and 811 is required to prime phosphorylation at other sites on Rb.[21] E2F1 then remains bound to Rb and is unavailable for cell cycle progression, leading to the observed strong antileukemic effects.

NRAS mutations activate MAPK/ERK signaling and have been associated with resistance to mIDH2 inhibitor treatment.[22, 23] Combination treatment with a mIDH1/2 inhibitor with azacitidine and inhibition of MAPK/ERK signaling may indeed target a specific dependency in IDH-mutated leukemias and may reduce the risk of relapse.

Although IDH1/2 mutations increase DNA methylation through the oncometabolite R-2HG,[24] IDH1/2 mutated leukemias were not associated with better response to azacitidine monotherapy in myelodysplastic syndrome and AML patients.[25, 26] We conclude that the synergistic effect of BAY1436032 and azacitidine derives from specific inhibition of MAPK/ERK and RB/E2F signaling. This is supported by the striking difference between sequential and simultaneous treatment with BAY1436032 and azacitidine. Simultaneous treatment during 5 days was 70-fold more efficient in eliminating LSCs than sequential treatment, suggesting improved efficacy of the combination when both drugs are present in the leukemic cell at the same time. This strongly argues for the concurrent application of both drugs in ongoing and future clinical trials. A phase 1 clinical study combining the IDH1 inhibitor ivosidenib with azacitidine is ongoing and

initial data showed promising results.[27] It may be worth investigating the effect of prolonged administration of azacitidine at a lower dose or with the oral formulation CC-486 concurrently with a mIDH1/2-inhibitor to further enhance its efficacy in IDH mutated AML patients.

In conclusion, the combination of BAY1436032 and azacitidine shows a strong reduction of LSCs by suppressing cell proliferation, self renewal and by inducing differentiation.

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Authors' Contributions

A.C. and M.H. conceived and designed the study. A.C., C.G., N.B., R.Go., K.G., R.S., B.O., J.W., and R.Ge. collected the data. A.C., C.G., R.Gab., and M.H. analyzed and assembled the data. S.K., O.P., M.W., M.J., and A.H. provided critical reagents. F.T., A.G. and M.H. collected patient samples and provided the patient data. A.C. and M.H. wrote the manuscript. All authors reviewed the data and edited and approved the final version of the manuscript.

Disclosure of Potential Conflicts of Interest

Chaturvedi and M. Heuser received research support to their University from Bayer AG. S. Kaulfuss, and M. Jeffers are employees of Bayer AG. M. Wagner, O. Panknin, and A. Haegebarth are employees and equity owners of Bayer AG. All other authors have no conflict of interest.

Materials and correspondence

Correspondence and material requests to be addressed to Michael Heuser or Anuhar Chaturvedi.

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Figure legends

Figure 1. Mutant IDH1 inhibitor BAY1436032 and azacitidine synergize to inhibit human IDH1 mutant AML cells ex vivo. (A) Inhibition of colony formation by combining BAY1436032 (100 nm, corresponding to the IC50 in previous experiments) with varying concentrations of azacitidine in colony forming assays using primary human AML cells with wildtype (wt) or mutant (mut) IDH1. The graph represents the proportion of colonies relative to cells treated with BAY1436032 at 100 nM (mean ± SEM). From the 6 patients with IDH1 mutant AML, 4 harbored an IDH1R132H mutation and one each an IDH1R132C and IDH1R132G mutation. (B) Proportion of viable cells in S phase of the cell cycle after treatment with BAY1436032 (100 nM) or azacitidine (100 nM) or the combination of both relative to DMSO-treated cells (mean ± SEM). From the 5 patients with IDH1 mutant AML, 3 harbored an IDH1R132H mutation and one each an IDH1R132C and IDH1R132G. (C) A representative FACS plot of IDH1wt and IDHmut primary AML cells treated ex vivo with either vehicle, BAY1436032, azacitidine or BAY1436032 and azacitidine in combination. (D) Inhibition of colony formation after treatment with serial dilutions of azacitidine and BAY1436032, alone or in combination using primary human IDH1mutant AML cells. Five patients harbored an IDH1R132H and one an IDH1R132C mutation. (E) Isobologram analysis of the combination of azacitidine and BAY1436032 in IDH1mut AML patient cells. The individual doses of azacitidine and BAY1436032 to achieve

90% growth inhibition (ED 90 or Fa = 0.9), 75% growth inhibition (ED 75 or Fa = 0.75), and 50% growth inhibition (ED 50 or Fa = 0.5) were plotted on the x- and y-axes. Combination index (CI) values calculated using CompuSyn software is depicted in the graph. A combination index (CI) of 1 indicates an additive effect, a CI < 1 a synergistic effect and a CI > 1 antagonism. ED-effective dose, Fa-fraction affected.

Figure 2. BAY1436032 synergizes with azacitidine to exert potent anti-leukemic activity in a patient-derived IDH1 mutant AML xenograft model in vivo. (A) Schematic representation of the treatment regimens; sim, simultaneous treatment with BAY1436032 and azacitidine; seq, sequential treatment with BAY1436032 and azacitidine. (B) Percentage of hCD45+ leukemic cells in peripheral blood of IDH1 mutant (R132C) PDX1 mice at different time points after treatment start with vehicle, azacitidine (1mg/kg, s.c., days 1-5 and days 29-34), BAY1436032 (150 mg/kg, p.o., continuously), or the sequential or simultaneous combination of BAY1436032 and azacitidine according to the treatment regimen shown in Figure 2A (mean ± SEM). (C) Percentage of hCD45+ leukemic cells in peripheral blood of individual mice transplanted with human IDH1 mutant AML cells and simultaneously treated with BAY1436032 and azacitidine. (D) White blood cell counts after different time points of treatment (mean ± SEM). (E) Platelet counts after different time points of treatment (mean ± SEM). (F) Kaplan–Meier survival curves of IDH1mutant PDX1 mice treated with vehicle, azacitidine (1 mg/kg, s.c.), BAY1436032 (150 mg/kg, p.o.), or the sequential or simultaneous combination of BAY1436032 and azacitidine according to the treatment regimen shown in Figure 2A. * P<0.5; **P<0.01; ***P<0.001.

Figure 3. Combination treatment with BAY 1436032 and azacitidine strongly depletes leukemia stem cells in vivo through inhibition of MAP-kinase signaling and activation of myeloid differentiation. (A) Limiting dilution transplantation of bone marrow cells from IDH1 mutant PDX mice treated with vehicle, azacitidine (1 mg/kg, s.c., days 1 to 5), BAY1436032 (150 mg/kg, p.o., q.d., 4 weeks) or the sequential or simultaneous combination of BAY1436032 and azacitidine with the same doses as in the single agent treated mice. 2,000,000, 200,000, 20,000, 2,000, 200 or 20 human AML cells per mouse were transplanted into 3 recipient mice per cell dose. LSC frequencies are shown (mean ± SEM, n = 3). Mice with hCD45+ cells in peripheral blood after 8 weeks (>0.1%) were scored positive. The log-fraction plot of the limiting dilution model has been generated using the ELDA software; the median stem cell frequencies are shown by solid lines and the 95% confidence intervals by dotted lines. The fold change in LSC frequency normalized to vehicle treated control mice is shown as a bar graph. The fold decrease in LSCs compared to vehicle treated mice is indicated on top of the bars. (B) Unsupervised hierarchical clustering using euclidean distance of cells from bone marrow of IDH1 mutant PDX mice treated with vehicle, azacitidine (1 mg/kg, s.c., days 1 to 5), BAY1436032 (150 mg/kg, p.o., q.d., 4 weeks) or the simultaneous combination of BAY1436032 and azacitidine. Cells were harvested from bone marrow at 4 weeks after treatment and sorted for hCD45+ cells. Gene expression profiling using RNA was performed on Affymetrix Human HG_U133 Plus 2.0 microarrays (n = 3 per group). (C) Principal component analysis of all treatment groups using the top 4000 differentially expressed genes. (D) Gene set enrichment analysis (MSigDB version 6.0) showing the most enriched transcription factor target gene sets from the indicated treatment comparisons. NES, normalized enrichment score. * gene sets involved in MAP Kinase signaling; ** gene sets involved in RB/E2F signaling (E) Heatmap from gene expression levels of MAP kinase signaling genes, RB/E2F signaling genes and myeloid differentiation genes from the bone marrow of IDH1 mutant PDX1 mice treated with vehicle, azacitidine (1 mg/kg, s.c., days 1 to 5), BAY1436032 (150 mg/kg, p.o., q.d., 4 weeks) or the

simultaneous combination of BAY1436032 and azacitidine. Gene expression was determined by quantitative RT-PCR relative to the housekeeping gene ABL and was normalized to gene expression in vehicle-treated cells (mean ± SEM, n=3 independent experiments). (F) Representative western blots of in vitro cultured HT1080, a fibrosarcoma cell line with an endogenous heterozygous IDH1R132C mutation treated with vehicle, azacitidine, BAY1436032 or the simultaneous combination of BAY1436032 and azacitidine using antibodies against the indicated signaling proteins. (G) Inhibition of colony formation by the MEK1/2 inhibitor trametinib in colony-forming cell assays using primary human AML cells with wildtype or mutant IDH1 (mean ± SEM). (H) Inhibition of colony formation by the CDK4/6 inhibitor abemaciclib in colony-forming cell assays using primary human AML cells with wildtype or mutant IDH1 (mean±SEM). From the six patients with IDH1 mutant AML, three harbored a IDH1R132H mutation and one each an IDH1R132C, IDH1R132L and IDH1R132G mutation. * P<0.5; *** P<0.001

Figure 4. Model of the combined activity of BAY1436032 and azacitidine on leukemia stem cells. BAY1436032 and azacitidine as single agents induce the expression of genes involved in myeloid differentiation (*PU.1, CEBPA,* and *GABPA*) and show additive effects in combination. MAP kinase signaling is synergistically inhibited by the combination treatment mediated by inhibition of ERK1/2 phosphorylation and suppression of its downstream targets (ELK1, ETS*,* CCND1). Suppression of the RB phosphorylation by the combined treatment of BAY1436032 and azacitidine suggests synergistic inhibition of the cyclinD1/CDK4 complex. Unphosphorylated retinoblastoma binds to E2F transcription factors and prevents the G1 to S transition of the cell cycle, thereby inhibiting cell proliferation.

Figure 1

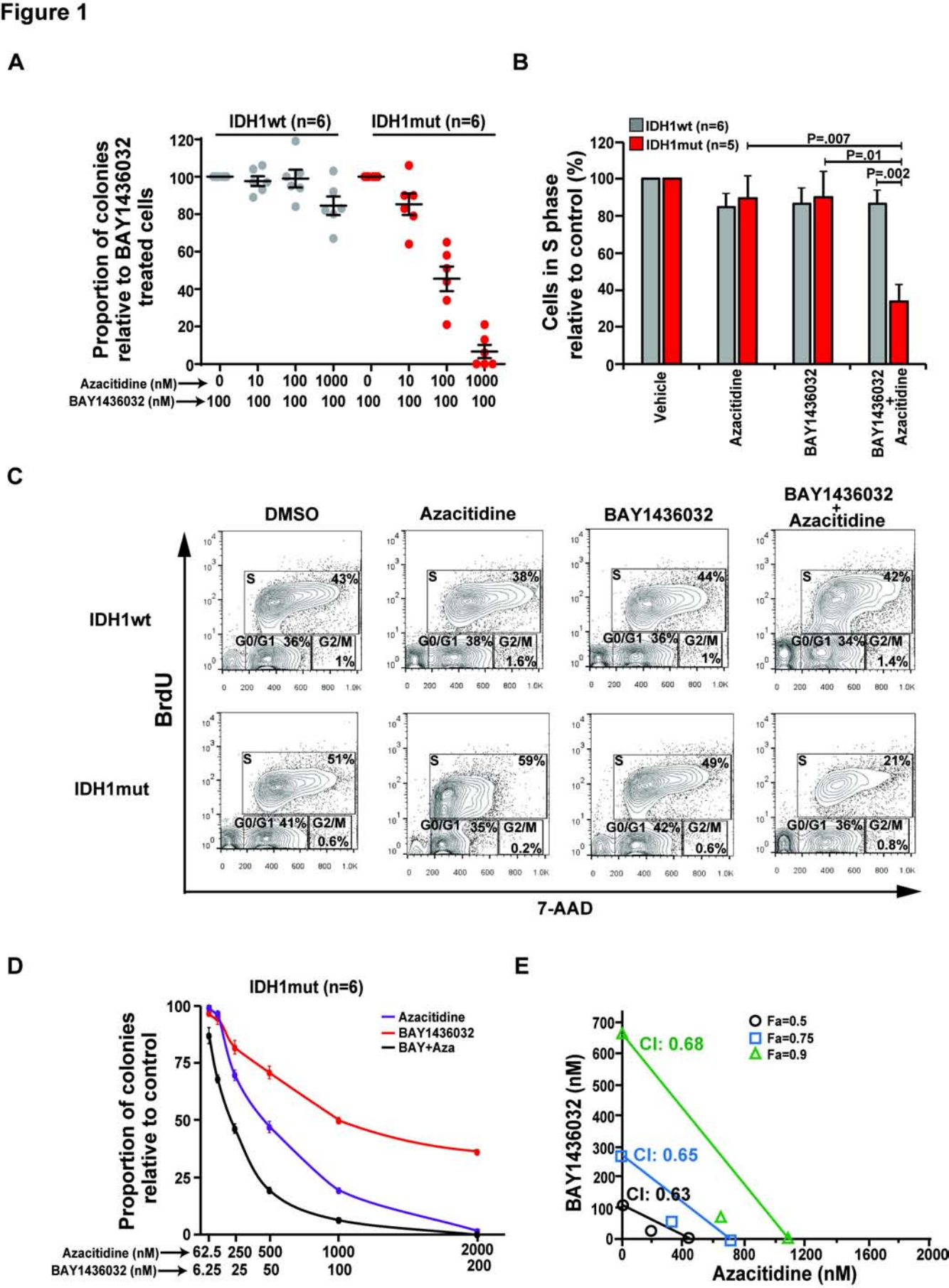

A
IDH1wt (n=6)
IDH1mut (n=6)
Proportion of colonies relative to BAY1436032 treated cells
Azacitidine (nM)
BAY1436032 (nM)
B
IDH1wt (n=6)
IDH1mut (n=5)
P=.007
P=.01
P=.002
Cells in S phase relative to control (%)
Vehicle
Azacitidine
BAY1436032
BAY1436032 + Azacitidine
C
DMSO
Azacitidine
BAY1436032
BAY1436032 + Azacitidine
IDH1wt
IDH1mut
BrdU
7-AAD
D
IDH1mut (n=6)
Azacitidine
BAY1436032
BAY+Aza
Proportion of colonies relative to control
Azacitidine (nM)
BAY1436032 (nM)
E
Fa=0.5
Fa=0.75
Fa=0.9
CI: 0.68
CI: 0.65
CI: 0.63
BAY1436032 (nM)
Azacitidine (nM)

Figure 2

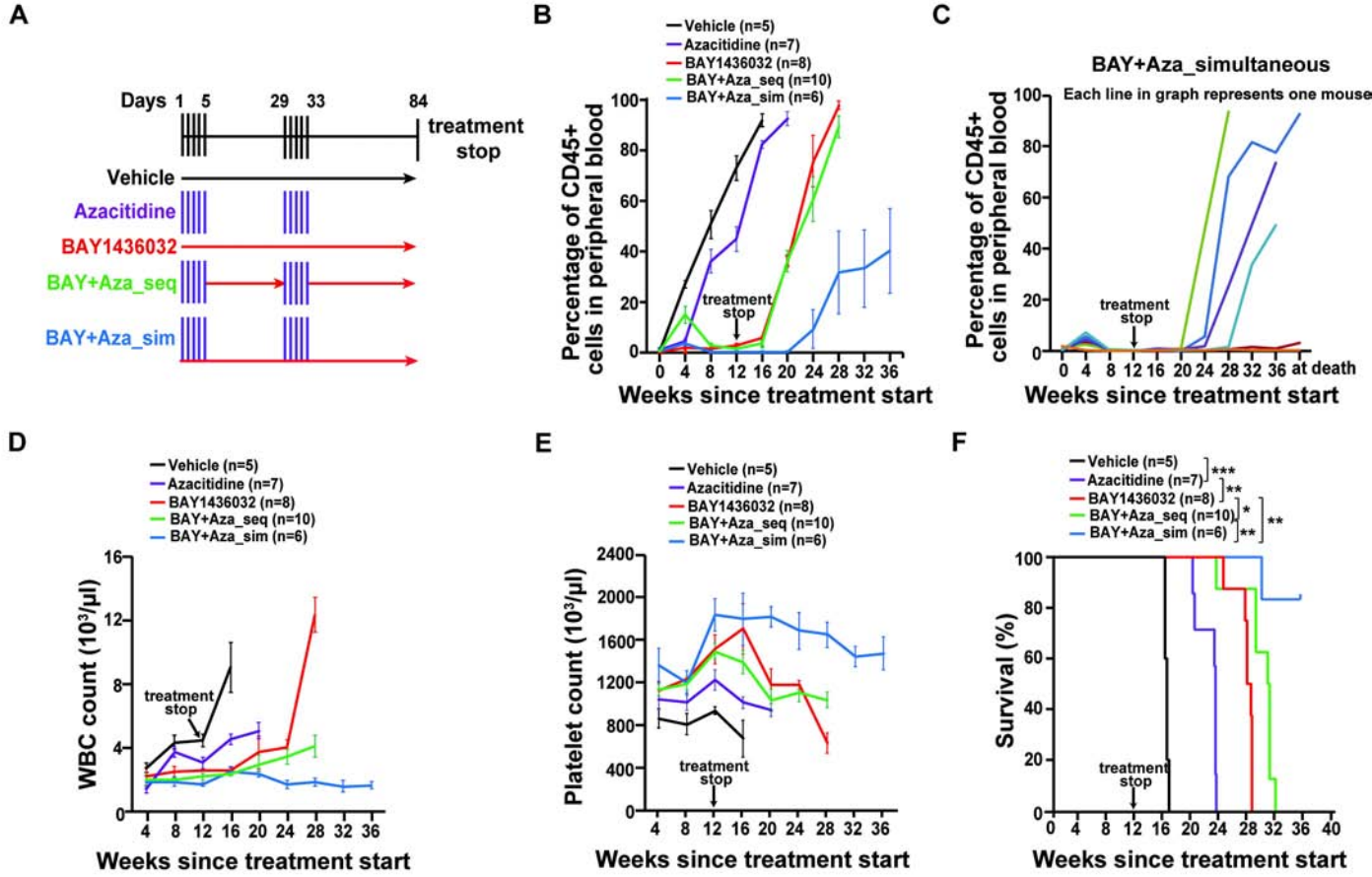
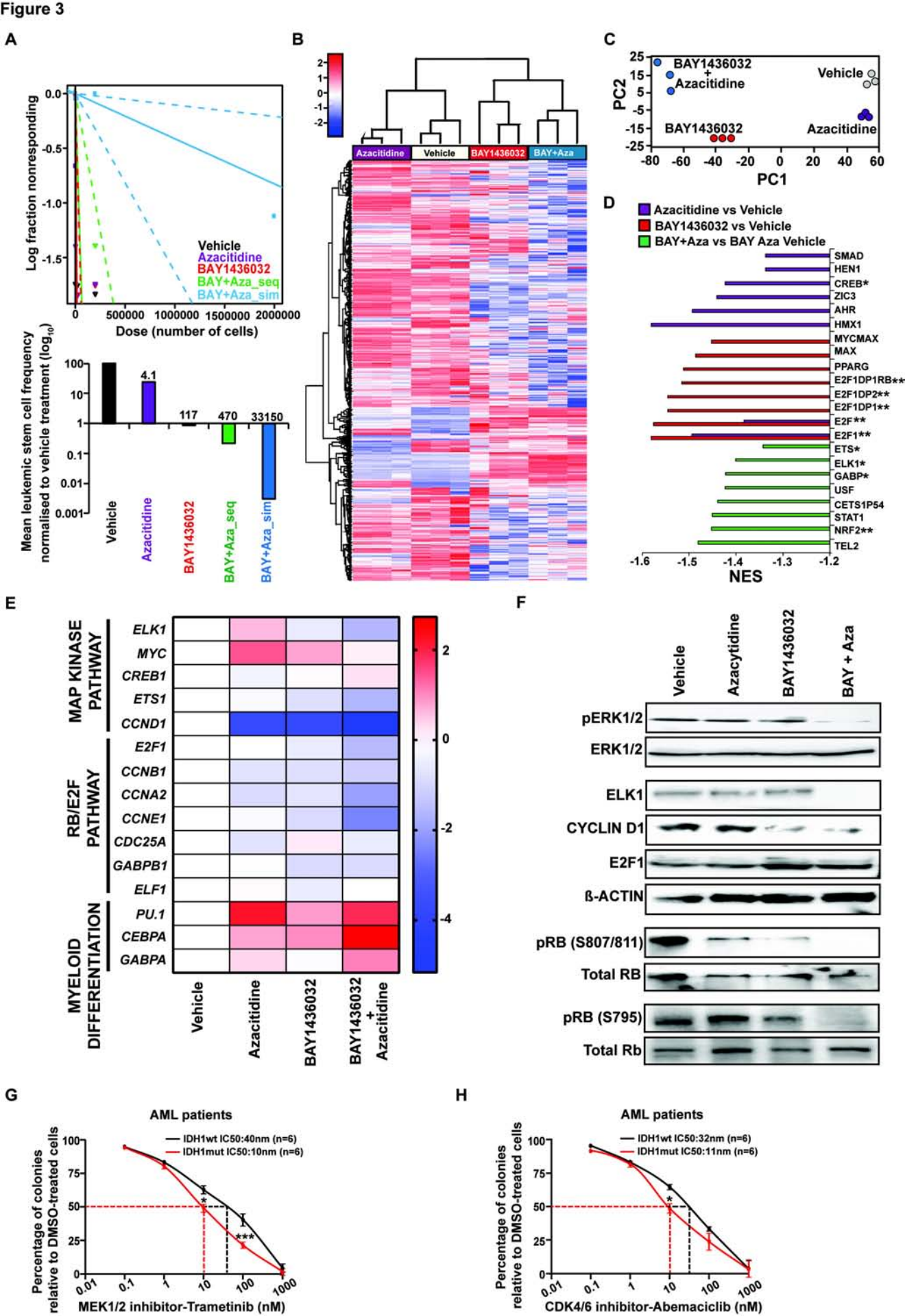
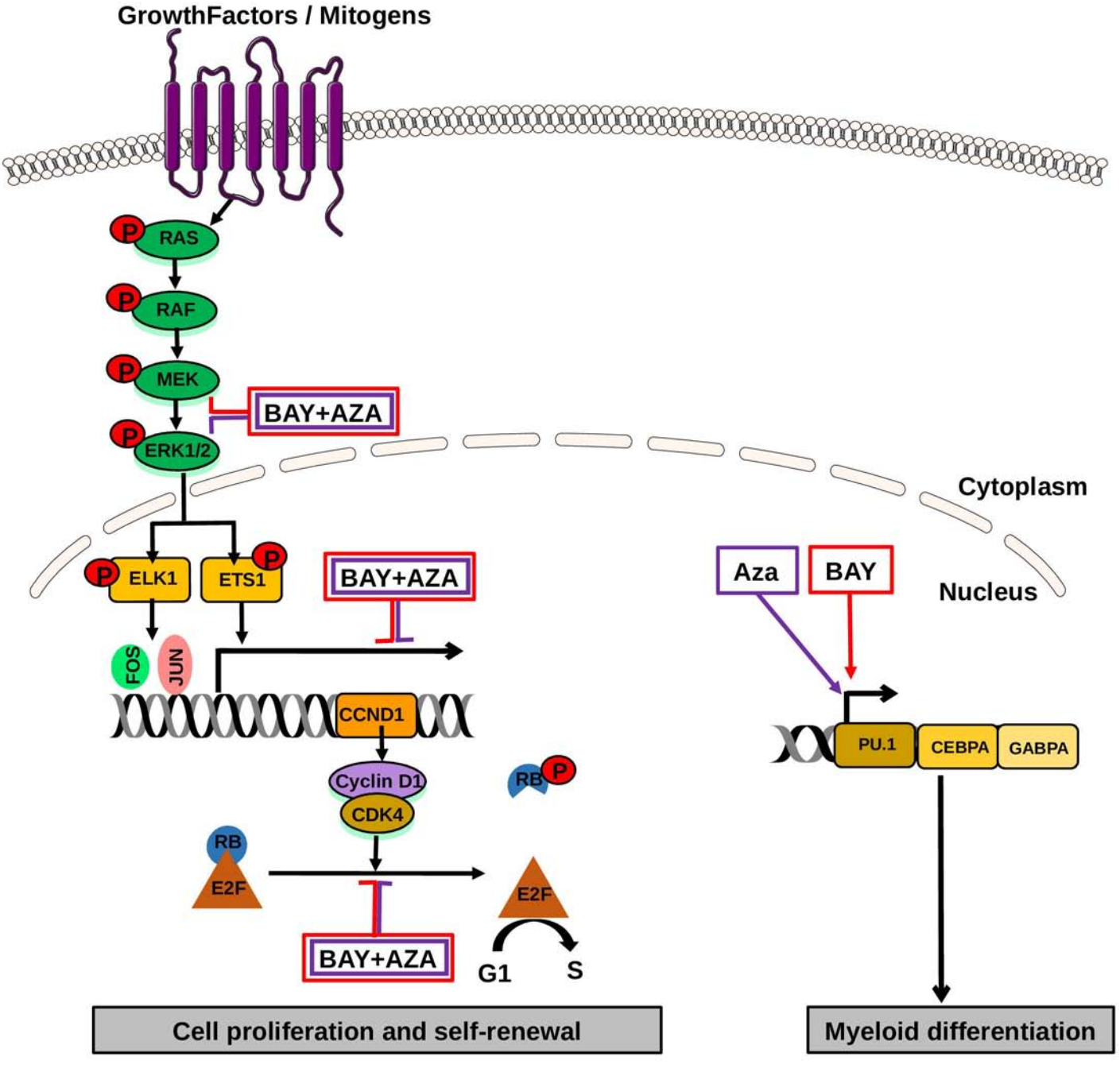

A
Days 1 5 29 33 84
treatment stop
Vehicle
Azacitidine
BAY1436032
BAY+Aza_seq
BAY+Aza_sim
B
Vehicle (n=5)
Azacitidine (n=7)
BAY1436032 (n=8)
BAY+Aza_seq (n=10)
BAY+Aza_sim (n=6)
Percentage of CD45+ cells in peripheral blood
treatment stop
Weeks since treatment start
C
BAY+Aza_simultaneous
Each line in graph represents one mouse
Percentage of CD45+ cells in peripheral blood
treatment stop
at death
Weeks since treatment start
D
Vehicle (n=5)
Azacitidine (n=7)
BAY1436032 (n=8)
BAY+Aza_seq (n=10)
BAY+Aza_sim (n=6)
WBC count (10³/µl)
treatment stop
Weeks since treatment start
E
Vehicle (n=5)
Azacitidine (n=7)
BAY1436032 (n=8)
BAY+Aza_seq (n=10)
BAY+Aza_sim (n=6)
Platelet count (10³/µl)
treatment stop
Weeks since treatment start
F
Vehicle (n=5)
Azacitidine (n=7)
BAY1436032 (n=8)
BAY+Aza_seq (n=10)
BAY+Aza_sim (n=6)
***
**
*
**
Survival (%)
treatment stop
Weeks since treatment start

Figure 3


A
Log fraction nonresponding
Vehicle
Azacitidine
BAY1436032
BAY+Aza_seq
BAY+Aza_sim
Dose (number of cells)
Mean leukemic stem cell frequency normalised to vehicle treatment (log10)
4.1
117
470
33150
B
Azacitidine
Vehicle
BAY1436032
BAY+Aza
C
BAY1436032 + Azacitidine
Vehicle
BAY1436032
Azacitidine
PC2
PC1
D
Azacitidine vs Vehicle
BAY1436032 vs Vehicle
BAY+Aza vs BAY Aza Vehicle
SMAD
HEN1
CREB*
ZIC3
AHR
HMX1
MYCMAX
MAX
PPARG
E2F1DP1RB**
E2F1DP2**
E2F1DP1**
E2F**
E2F1**
ETS*
ELK1*
GABP*
USF
CETS1P54
STAT1
NRF2**
TEL2
NES
E
MAP KINASE PATHWAY
ELK1
MYC
CREB1
ETS1
CCND1
RB/E2F PATHWAY
E2F1
CCNB1
CCNA2
CCNE1
CDC25A
GABPB1
ELF1
MYELOID DIFFERENTIATION
PU.1
CEBPA
GABPA
Vehicle
Azacitidine
BAY1436032
BAY1436032 + Azacitidine
F
Vehicle
Azacytidine
BAY1436032
BAY + Aza
pERK1/2
ERK1/2
ELK1
CYCLIN D1
E2F1
ß-ACTIN
pRB (S807/811)
Total RB
pRB (S795)
Total Rb
G
AML patients
IDH1wt IC50:40nm (n=6)
IDH1mut IC50:10nm (n=6)
Percentage of colonies relative to DMSO-treated cells
MEK1/2 inhibitor-Trametinib (nM)
H
AML patients
IDH1wt IC50:32nm (n=6)
IDH1mut IC50:11nm (n=6)
Percentage of colonies relative to DMSO-treated cells
CDK4/6 inhibitor-Abemaciclib (nM)


GrowthFactors / Mitogens
RAS
RAF
MEK
ERK1/2
BAY+AZA
Cytoplasm
ELK1
ETS1
BAY+AZA
Aza
BAY
Nucleus
FOS
JUN
CCND1
PU.1
CEBPA
GABPA
Cyclin D1
CDK4
RB
RB
E2F
E2F
BAY+AZA
G1
S
Cell proliferation and self-renewal
Myeloid differentiation

Synergistic Activity of IDH1 Inhibitor BAY1436032 with Azacitidine in IDH1 Mutant Acute Myeloid Leukemia

Supplementary information

Supplementary methods

Compound Preparation

For *in vivo* administration 30 mg/ml of BAY1436032 was prepared in 2-(hydroxypropyl)-β-cyclodextrin (HP-ß-CD, Sigma-Aldrich, Munich, Germany). In brief, 600 mg BAY1436032 was added to 20 ml of 30% HP-ß-CD, pH was made alkaline with 50-100 µl of 10 M NaOH, followed by sonication (Bioruptor, Diagenode, Seraing, Belgium) with 15 seconds pulse on and 15 seconds pulse off until the drug was completely dissolved. Finally, the pH was readjusted to 7.4 with 2 M HCl. The fresh solution was prepared every week for *in vivo* administration. Azacitidine (Celgene, Munich, Germany) diluted in 0.9% NaCl, was purchased from the Pharmacy of Hannover Medical School, and administered within two hours after preparation to the mice. For *in vitro* studies a 1 mM stock solution of BAY1436032, 1 mM of trametinib (Selleckchem, Biozol diagnostics, Eching, Germany), and 1 mM of abemaciclib (Selleckchem) was prepared in DMSO or a 1 mM stock solution of azacitidine was prepared in 0.9% NaCl and stored at -20°C for a maximal period of two weeks. All dilutions were freshly prepared in cell culture medium and used immediately.

Limiting dilution transplantation

For limiting dilution transplantation, primary mice with established leukemia (the proportion of human AML cells in peripheral blood at the start of treatment was 70-80%) were treated for 4 weeks with either vehicle, azacitidine, BAY1436032, or the sequential or simultaneous

combination of BAY1436032 and azacitidine. After 4 weeks of treatment, bone marrow cells were obtained from primary mice and injected intravenously at doses of 2,000,000, 200,000, 20,000, 2,000, 200 or 20 cells (n = 3 mice/dose) into irradiated secondary NSG recipients. Eight weeks after transplantation, the presence of transplanted human cells in peripheral blood was assessed by flow cytometry. Engraftment was determined positive when more than 0.1% $hCD45^+$ cells were detected. The frequency of leukemia initiating cells within the human CD45+ AML cell population was calculated by applying Poisson statistics to the proportion of negative recipients at different dilutions using the ELDA software.[1]

Clonogenic progenitor assay

Colony-forming cell units were assayed in methylcellulose (Methocult H4100; StemCell Technologies, Cologne, Germany) supplemented with 10 ng/mL IL3, 10 ng/mL GM-CSF, 50 ng/mL SCF, 50 ng/mL FLT3-ligand and 3 U/mL EPO (PeproTech, Hamburg, Germany). BAY1436032 and/or azacitidine were 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.

Cell culture conditions, cell cycle, and apoptosis

Patient-derived AML cells, freshly isolated or cryopreserved, were cultured in IMDM medium (StemCell Technologies, Cologne, Germany) supplemented with 10% FBS (Sigma-Aldrich, Munich, Germany), 2 mM L-Glutamine (Gibco, Thermofisher Scientific, Bremen, Germany), 20 ng/ml human IL-3, 20 ng/ml human IL-6, 20 ng/ml human GMCSF, 20 ng/ml human GCSF and 50 mg/ml human SCF (all from PeproTech, Hamburg, Germany) and were incubated at 37°C with 5% CO_2 in the humidified atmosphere. Treatment was carried out with 100 nM BAY1436032 and 100 nM azacitidine. 60 hours after the treatment 10 µM of BrdU was added to the cells for 12 hours. Cell cycle analysis was performed according to the manufacturer's

protocol (BD Pharmingen Cat no. 557892). $2x10^5$ cells were acquired per sample. Cell cycle phases were determined according to standard procedures, where BrdU positive cells are in the S phase of the cell cycle. For apoptosis measurement cells were stained with Annexin V-APC and 7AAD after 72 hours of treatment according to the manufacturer's protocol (BD Pharmingen Cat no. 560931) and analyzed on a BD FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Annexin V single positive and Annexin V/7AAD double positive cells were combined to represent apoptotic cells.

Antibodies for flow cytometry

The monoclonal antibody used were CD45-FITC (Clone HI30, Cat no.555482, lot no.7137972), CD14-APC (Clone M5E2, cat no. 555399, Lot no. 7116537) and CD15-PE (Clone W6D3, cat no. 562371) from BD Biosciences, Heidelberg, Germany. Data were collected on a FACS Calibur (BD Biosciences, Heidelberg, Germany).

Gene expression profiling using microarrays

For gene expression profiling RNA was extracted using the RNeasy Plus mini kit (Qiagen) from $hCD45^+$ cells that were sorted from bone marrow of PDX mice 4 weeks after treatment with vehicle, azacitidine (1 mg/kg, s.c., days 1 to 5), BAY1436032 (150 mg/kg, p.o., q.d., 4 weeks) or the simultaneous combination of BAY1436032 and azacitidine.

Quality and integrity of total RNA was controlled on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). 500 ng of total RNA was used for biotin labeling according to the 3´ IVT Express Kit (Affymetrix/ThermoFisher Scientific, Braunschweig, Germany). 7.5 µg of biotinylated cRNA was fragmented and placed in a hybridization cocktail containing 4 biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix GeneChip HG-U133 2.0 for 16 hours at 45°C. Steps for washing and SA-PE staining were processed on the fluidics

station 450 using the recommended FS450 protocol (Affymetrix). Image Analysis was performed on the GCS3000 Scanner and GCOS1.2 Software Suite (Affymetrix).

Microarray data was analyzed and presented using R packages and Cluster 3.0 software. [2] Arrays were background corrected, normalized and summarized with the *rma* function of the *affy* package [3]. The *limma* package [4] was used to select differentially expressed genes;the *gplots* package was used for drawing heatmaps. Principal component analysis (PCA) was done with Cluster 3.0 software. Expression values of the top 4000 differentially regulated genes in all arrays were selected for PCA. Expression of the top 500 differentially regulated genes were used for unsupervised hierarchical clustering, the results of which were visualized as a heatmap in Figure 3B. The heatmap in Supplementary Figure S6 used 89 genes with available Affymetrix probeset ID that were differentially expressed between LSC+ and LSC- cell fractions from AML patients (known as the LSC17 gene signature).[5]

The Broad Institute GSEA software package was employed for gene set enrichment analysis using gene ontology gene sets from the Molecular Signatures Database (http://www.broad.mit.edu/ gsea/msigdb/). Gene expression profiling data can be found at the gene expression omnibus database under GEO accession number GSE122428.

Quantification of allele frequencies of mutant IDH1 in vivo

Mutated allele frequencies in blood samples from PDX1 mice were determined using the previously developed MRD protocol.[6] We designed primers to amplify a 114 bp cDNA fragment around the IDH1 mutation site. The fragments were sequenced with the Illumina Miseq sequencer. Paired-end output sequences/reads were aligned to the IDH1 cDNA sequence after combining overlapping forward and reverse reads. Sequences having mouse-specific alterations (there were 11 such positions on the amplicon) were removed. The variant allele frequency was calculated as a ratio of the number of mutated sequences to the number of all aligned sequences.

Gene expression profiling by RNA sequencing

Human AML cells isolated from the spleen and bone marrow of IDH1 R132C (PDX1) mice were cultured in IMDM medium (Stemcell Technology Inc. Vancouver, Canada) supplemented with 10% FBS (Sigma-Aldrich, Munich, Germany), 2 mM L-Glutamine (Life Technologies, Karlsruhe, Germany), 1% Penicillin/Streptomycin (Gibco, ThermoFischer Scientific, Braunschweig, Germany) , 20 ng/ml human GM-CSF, 20 ng/ml human G-CSF, 20 ng/ml human IL-6, 50 ng/µl, human SCF and 20 ng/µl human IL-3 (all from PeproTech, Hamburg, Germany) and were incubated at 37 °C with 5% CO_2 in humidified atmosphere. Treatment was carried out with DMSO (vehicle), 500 nM azacitidine daily from day 1 to day 4, 50 nM BAY1436032 daily from day 1 to day 5, the sequential combination (azacitidine daily from day 1 to day 4 and BAY1436032 daily from day 5 to day 9) and the simultaneous combination (azacitidine daily from day 1 to day 4 and BAY1436032 daily from day 1 to day 5). RNA was isolated on day 6 for cells treated with vehicle, azacitidine, BAY1436032 and the simultaneous combination and on day 10 for the sequential combination using the AllPrep DNA/RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted at 20 µl H2O, and concentration and purity were measured with the NanoDrop 2000 spectrophotometer (ThermoScientific, Darmstadt, Germany). Approximately 150 ng from each sample were sequenced on the Illumina NovaSeq 6000 system with paired-end reads of 50 bp in size. Sequences were aligned to the human genome hg19 with the help of Gencode v.n 25 transcriptome annotations.[7] RNAseq expression data was aligned to GRCh38 / Gencode v.32. A larger dataset of annotated transcripts was used, when more appropriate.[8] The main alignment program was STAR;[9] the program bowtie2 [10] was used for comparison. Mapping to the transcriptome and gene expression analysis was done with TopHat and Cufflinks [11] with the help of Gencode annotation of genes and alternative transcript variants. Gene and transcript expression was quantified by FPKM (Fragments Per Kilobase per Million), TPM (Transcripts Per

Million) values, as well as by fragment counts. The latter was calculated from cufflinks outputs using the program HTSeq [12] and used as input to DESeq2 analysis.[13]

DNA Methylation Microarrays

Genomic DNA (500 ng) isolated from the same samples that were used for RNA sequencing analysis above was bisulfite-treated using the EZ-96 DNA Methylation-Lightning MagPrep Kit (Zymo Research, Freiburg, Germany) according to the manufacturer's instructions. DNA methylation was assessed using the Infinium MethylationEPIC BeadChip Kit (Illumina, San Diego, USA) following the Infinium HD Methylation Assay protocol. Subsequent steps (i.e., amplification, fragmentation, extension, hybridization, staining, and scanning) were performed at the Life & Brain Center (Bonn, Germany). Raw data were processed and quality checked using Illumina's GenomeStudioV2011.1 methylation module (v.1.8).

Cell line

The HT-1080 cell line was purchased from ATCC (ATCC® CCL-121™, Manassas, Virginia, United States) and cultured in Minimum Essential Media (Gibco, ThermoFischer Scientific, Schwerte, Germany). The cells were negative for mycoplasma during routine mycoplasma testings in the lab.

Quantitative RT-PCR

RNA was extracted, reverse transcribed and quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using SYBR green (Invitrogen, ThermoFischer Scientific, Schwerte, Germany) for quantification of double stranded DNA on a StepOne Plus cycler (Applied Biosystems, Darmstadt, Germany). Relative expression was determined with the $2^{-\Delta\Delta CT}$ method, and the housekeeping gene transcript *Abl1* and the gene expression ratio of drug-treated cells relative to vehicle-treated cells was used to normalize the results.

Primers of quantitative PCR

*ELK1*_forward primer	5'-CAGCCAGAGGTGTCTGTTACC-3'
*ELK1*_reverse primer	5'-GAGCGCATGTACTCGTTCC-3'
*MYC*_ forward primer	5'-GGCTCCTGGCAAAAGGTCA-3'
*MYC*_ reverse primer	5'-CTGCGTAGTTGTGCTGATGT-3'
*CREB1*_ forward primer	5'-ATTCACAGGAGTCAGTGGATAGT-3'
*CREB1*_ reverse primer	5'-CACCGTTACAGTGGTGATGG-3'
*ETS1*_ forward primer	5'-GATAGTTGTGATCGCCTCACC-3'
*ETS1*_ reverse primer	5'-GTCCTCTGAGTCGAAGCTGTC-3'
*CCND1*_ forward primer	5'-GCTGCGAAGTGGAAACCATC-3'
*CCND1*_ reverse primer	5'-CCTCCTTCTGCACACATTTGAA-3'
*E2F1*_ forward primer	5'-ACGCTATGAGACCTCACTGAA-3'
*E2F1*_ reverse primer	5'-TCCTGGGTCAACCCCTCAAG-3'
*CCNB1*_ forward primer	5'-AATAAGGCGAAGATCAACATGGC-3'
*CCNB1*_ reverse primer	5'-TTTGTTACCAATGTCCCCAAGAG-3'
*CCNA2*_ forward primer	5'-CGCTGGCGGTACTGAAGTC-3'
*CCNA2*_ reverse primer	5'-GAGGAACGGTGACATGCTCAT-3'
*CCNE1*_ forward primer	5'-AAGGAGCGGGACACCATGA-3'
*CCNE1*_ reverse primer	5'-ACGGTCACGTTTGCCTTCC-3'
*CDC25A*_ forward primer	5'-GTGAAGGCGCTATTTGGCG-3'
*CDC25A*_ reverse primer	5'-TGGTTGCTCATAATCACTGCC-3'
*GABPB1*_ forward primer	5'-TCCACTTCATCTAGCAGCACA-3'
*GABPB1*_ reverse primer	5'-GTAATGGTGTTCGGTCCACTT-3'
*ELF1*_ forward primer	5'-TGTCCAACAGAACGACCTAGT-3'
*ELF1*_ reverse primer	5'-GGCAGGAAAAATAGCTGGATCAC-3'

*PU.1*_ forward primer	5'-GTGCCCTATGACACGGATCTA-3'
*PU.1*_ reverse primer	5'-AGTCCCAGTAATGGTCGCTAT-3'
*CEBPA*_ forward primer	5'-TTCACATTGCACAAGGCACT-3'
*CEBPA*_ reverse primer	5'-GAGGGACCGGAGTTATGACA-3'
*GABPA*_ forward primer	5'-TTAAACCTGCGGACACTGTTG-3'
*GABPA*_ reverse primer	5'-GTATCCCAAGGCGTTCTTGTT-3'

Immunoblotting

HT-1080 cells were treated with either Vehicle or 1 µM of BAY1436032 or 1 µM of azacitidine alone or in combination at 0 hours and 24 hours. 2 hours after the second treatment, 5 million cells were collected, washed with PBS, resuspended in RIPA buffer (cat no. 89901, Thermo Fischer Scientific, Bremen, Germany), supplemented with the HALT protease inhibitor cocktail (cat no. 78430, Thermo Fischer Scientific, Bremen, Germany) and lysed on ice for 20 minutes with gentle agitation. After centrifugation at 10,000 rpm for 10 minutes at 4°C, the supernatant was collected and the protein concentration was estimated using the Pierce BCA protein assay kit (Thermo Fischer Scientific, Bremen, Germany). 30 µg of protein/sample was separated by SDS PAGE, transferred to a PVDF membrane, blocked and immunoblotted. Clarity Western ECL Substrate (Biorad, Munich, Germany) was used for chemiluminescent protein detection with a ChemiDoc MP Imaging System (Bio-Rad, Munich, Germany).

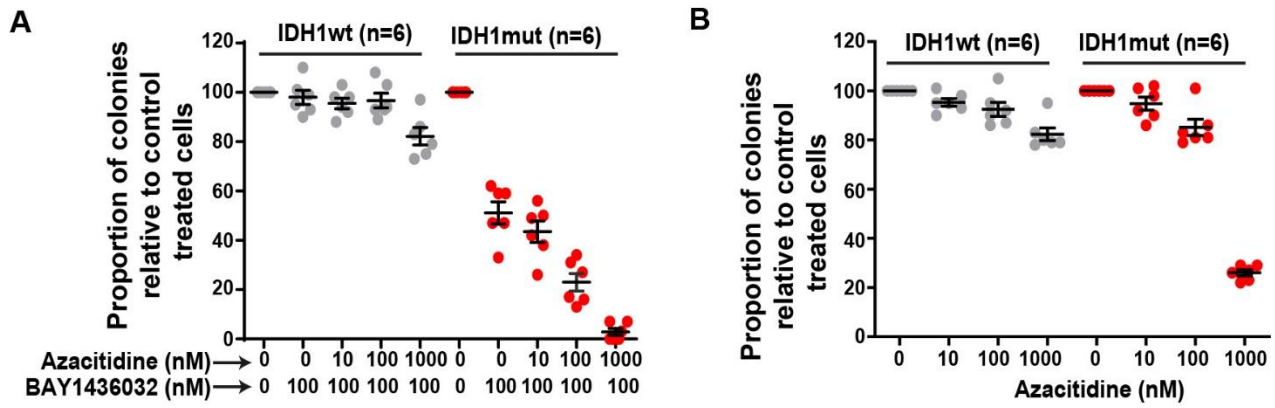
Antibodies for Immunoblotting

The antibodies used for immunoblotting are anti-phosho ERK1/2 (clone: D13.14.4E, cat no. 4370), anti-ERK1/2 (cat no. 9102), anti-ß-actin (clone: 13E5, cat no. 4970) and anti-phospho S807/811 Rb (Cat no.9308) from Cell Signaling Technology, Frankfurt, Germany. Anti-phospho S795 Rb (cat no. ab47474) from Abcam, Cambridge, UK. Anti-ELK1 (clone:E-5, cat no. sc-365876), anti-CyclinD1 (clone:DCS-6, cat no. sc-20044), anti-E2F1 (clone: C-20, cat no. sc-193)

and anti-Rb (clone: M-153, cat no. sc-7905) from Santa Cruz Biotechnology, Heidelberg, Germany.

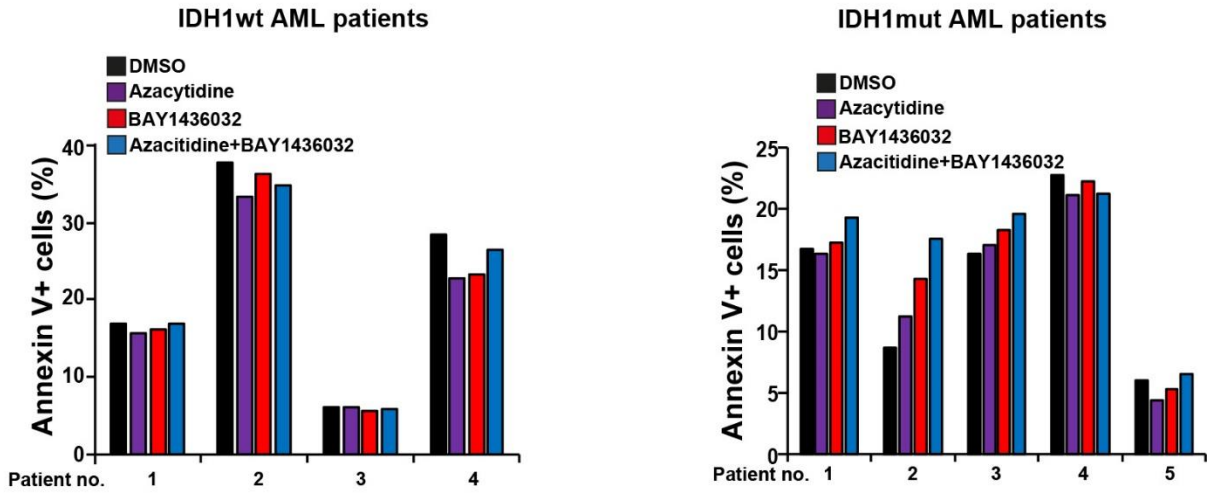
Supplementary Figures

Figure S1



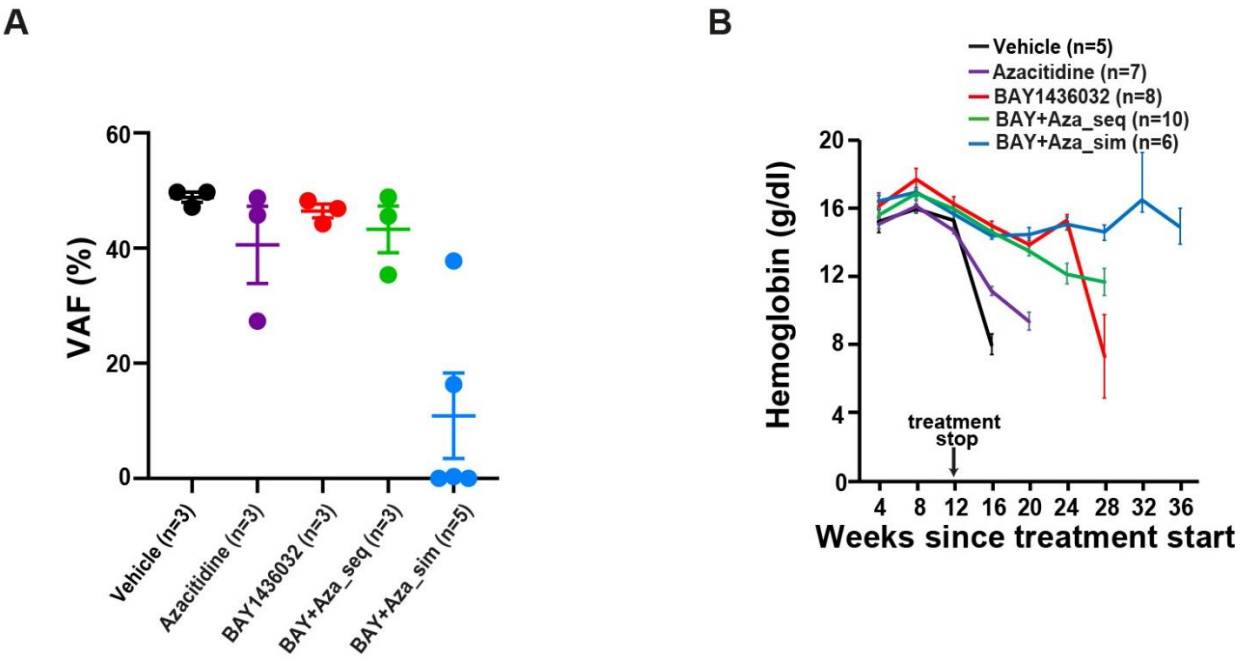
Supplementary Figure S1. Synergistic activity of IDH1 inhibitor BAY1436032 and azacitidine in IDH1 mutant but not in IDH1 wildtype human AML cells relative to control treated cells. (A) Inhibition of colony formation by the simultaneous combination treatment of BAY1436032 (100 nM, corresponding to the IC_{50} in previous experiments) with varying concentrations of azacitidine in colony forming assays using primary human AML cells with wildtype or mutant IDH1. (B) Inhibition of colony formation with varying concentrations of azacitidine in colony forming assays using primary human AML cells with wildtype or mutant IDH1. The graph represents the proportion of colonies relative to DMSO treated cells (mean ± SEM). From the 6 patients with IDH1 mutant AML, 4 harbored an I*DH1R132H* mutation and one each an *IDH1R132C* and *IDH1R132G* mutation.

Figure S2



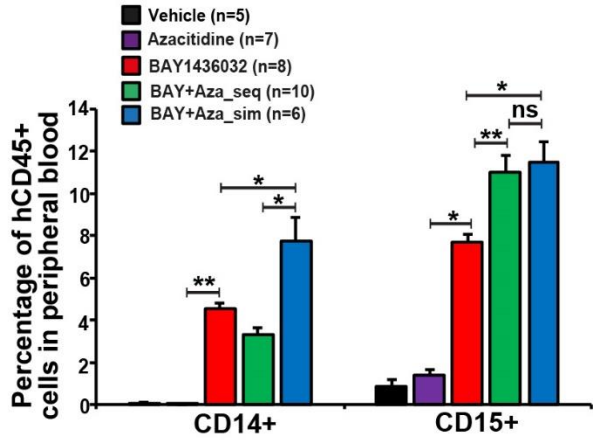
Supplementary Figure S2. The percentage of apoptotic cells did not differ between single agent and combination treatments. Percentage of Annexin V positive cells after treatment with BAY1436032 (100 nM) or azacitidine (100 nM) or the combination of both in IDH wildtype (right) and IDH1 mutant (left) primary AML patient cells. From the 5 patients with IDH1 mutant AML, 3 harbored an *IDH1R132H* mutation and one each an *IDH1R132C* and *IDH1R132G* mutation.

Figure S3



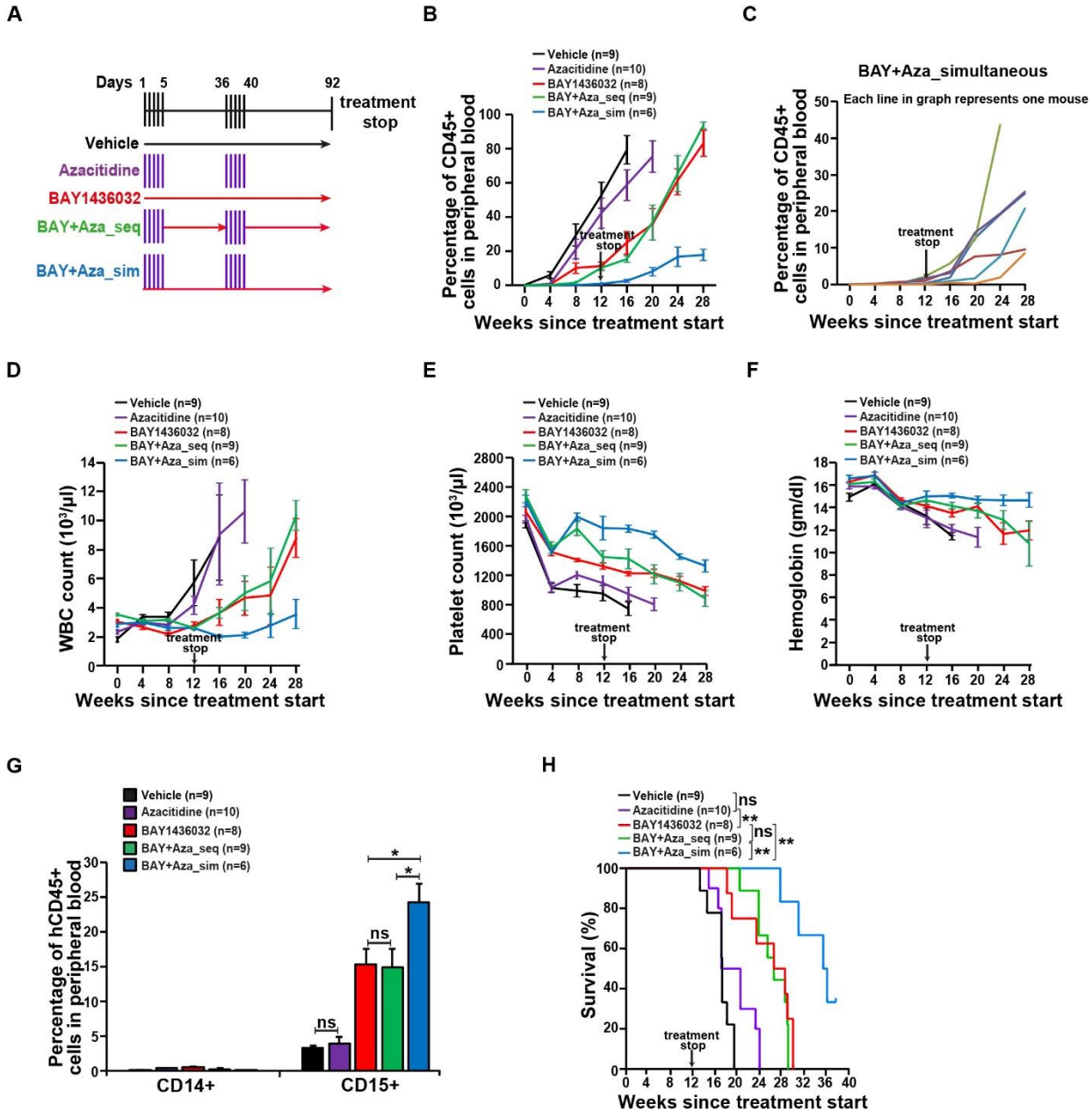
Supplementary Figure S3. IDH1mutant allele fraction is decreased and hemoglobin levels are maintained by the combined simultaneous treatment with BAY1436032 and azacitidine. (A) IDH1 mutant allele frequency assessed by next generation sequencing in peripheral blood of IDH1 mutant PDX mice at 8 weeks after the stop of treatment (20 weeks since treatment start) mean ± SEM for the indicated number of mice. (B) Hemoglobin in the peripheral blood of IDH1 mutant PDX mice at different time points after treatment start with vehicle, azacitidine (1 mg/kg; s.c., days 1-5 and days 29-33), BAY 1436032 (150 mg/kg, p.o., q.d., continuously), or the sequential or simultaneous combination of BAY1436032 and azacitidine according to the treatment regimen shown in Figure 2A.

Figure S4



Supplementary Figure S4: The combination of BAY1436032 and azacitidine has additive effects on differentiation of *IDH1* mutant AML cells treated in vivo. Percentage of myeloid differentiation markers CD14 and CD15 from hCD45+ cells in peripheral blood of *IDH1*mutant (R132C) PDX1 mice at 4 weeks after the start of treatment with vehicle, azacitidine (1mg/kg, s.c., days 1-5), BAY1436032 (150 mg/kg, p.o., continuously), or the sequential or simultaneous combination of BAY1436032 and azacitidine (mean ± SEM). * P<0.5; **P<0.01; ns=non-significant

Figure S5

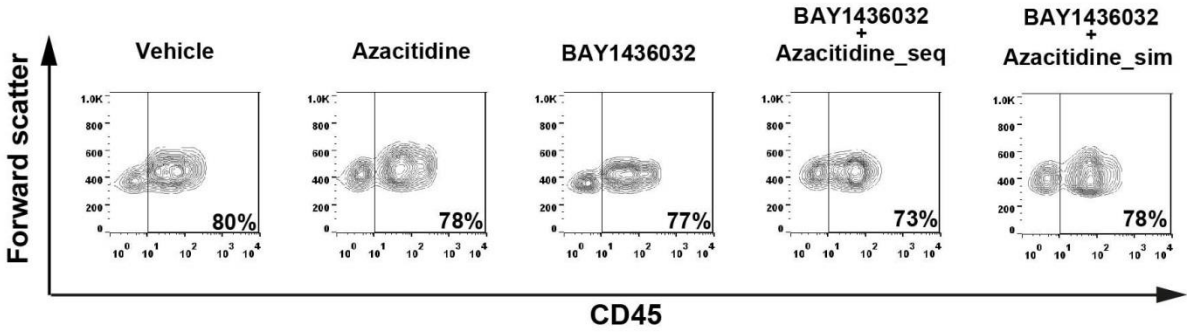


Supplementary Figure S5. BAY1436032 synergizes with azacitidine to exert potent anti-leukemic activity in an independent patient-derived IDH1 mutant AML xenograft model in vivo (PDX2). (A) Schematic representation of the treatment regimens; sim, simultaneous treatment with BAY1436032 and azacitidine; seq, sequential treatment with BAY1436032 and azacitidine. (B) Percentage of hCD45+ leukemic cells in peripheral blood of IDH1 mutant (R132H) PDX2 mice at different time points after treatment start with vehicle, azacitidine

(1mg/kg, s.c., days 1-5 and days 36-40), BAY1436032 (150 mg/kg, p.o., continuously), or the sequential or simultaneous combination of BAY1436032 and azacitidine according to the treatment regimen shown in Figure S5A (mean ± SEM). (C) Percentage of hCD45+ leukemic cells in peripheral blood of individual mice transplanted with human IDH1 mutant AML cells and simultaneously treated with BAY1436032 and azacitidine. (D) White blood cell counts after different time points of treatment (mean ± SEM). (E) Platelet counts after different time points of treatment (mean ± SEM). (F) Hemoglobin after different time points of treatment (mean ± SEM). (G) Kaplan–Meier survival curves of IDH1 mutant PDX2 mice treated with vehicle, azacitidine (1 mg/kg, s.c.), BAY1436032 (150 mg/kg, p.o.), or the sequential or simultaneous combination of BAY1436032 and azacitidine according to the treatment regimen shown in Figure S5A. (H) Percentage of myeloid differentiation markers CD14 and CD15 from hCD45+ cells in peripheral blood of IDH1 mutant (R132H) PDX2 mice at 12 weeks after the start of treatment. (mean ± SEM). * $P<0.5$; ns=non-significant

Figure S6

A

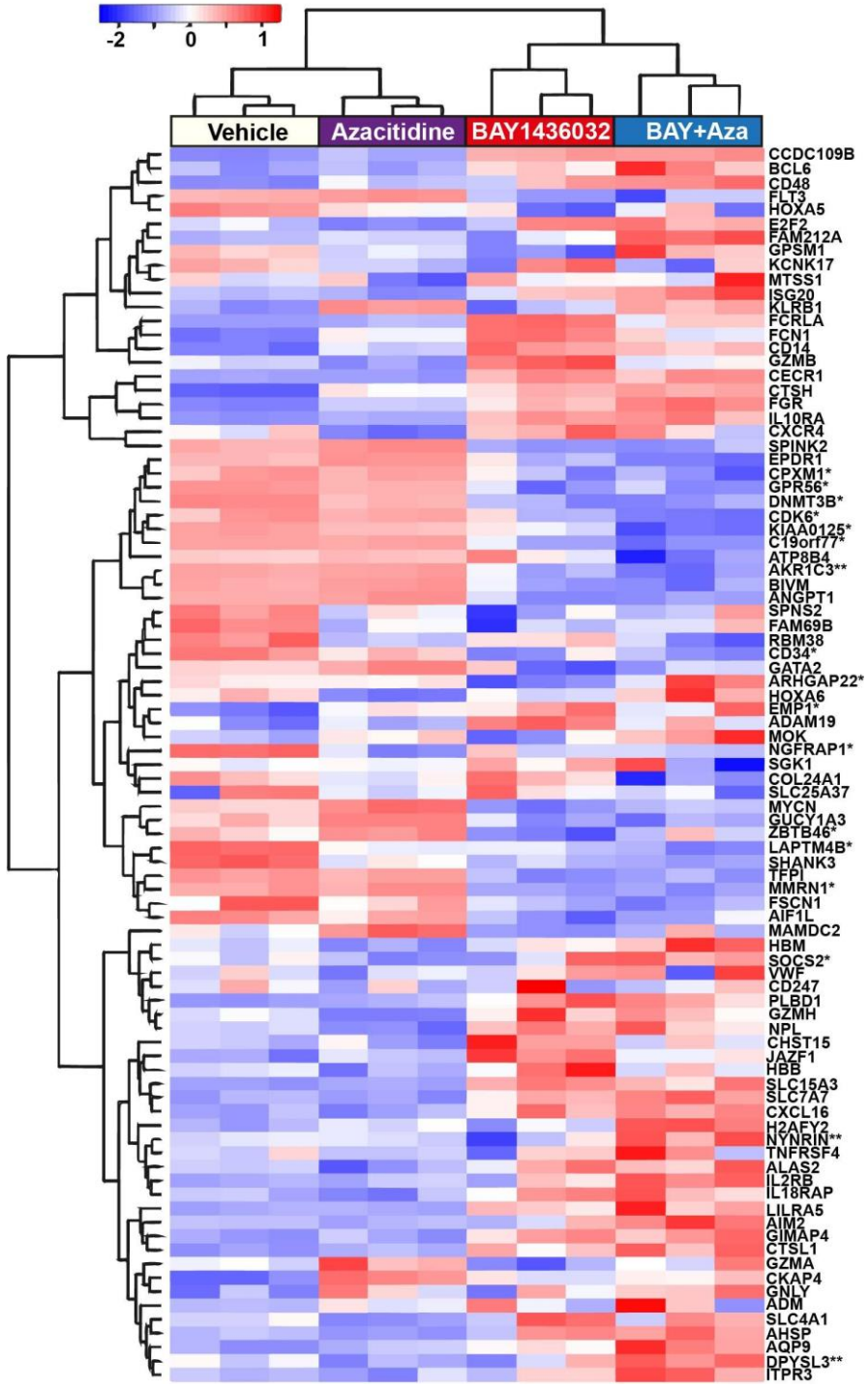


B

Group	Stem cell frequency	±SEM	P Value
Vehicle	1/73	18-287	0.2
Azacitidine	1/304	48-1,994	0.005
BAY1436032	1/8,580	2,440-30,173	0.2
BAY+Aza_seq	1/34,300	5,913-199,114	0.0002
BAY+Aza_sim	1/2,420,000	612,820-9,558,998	

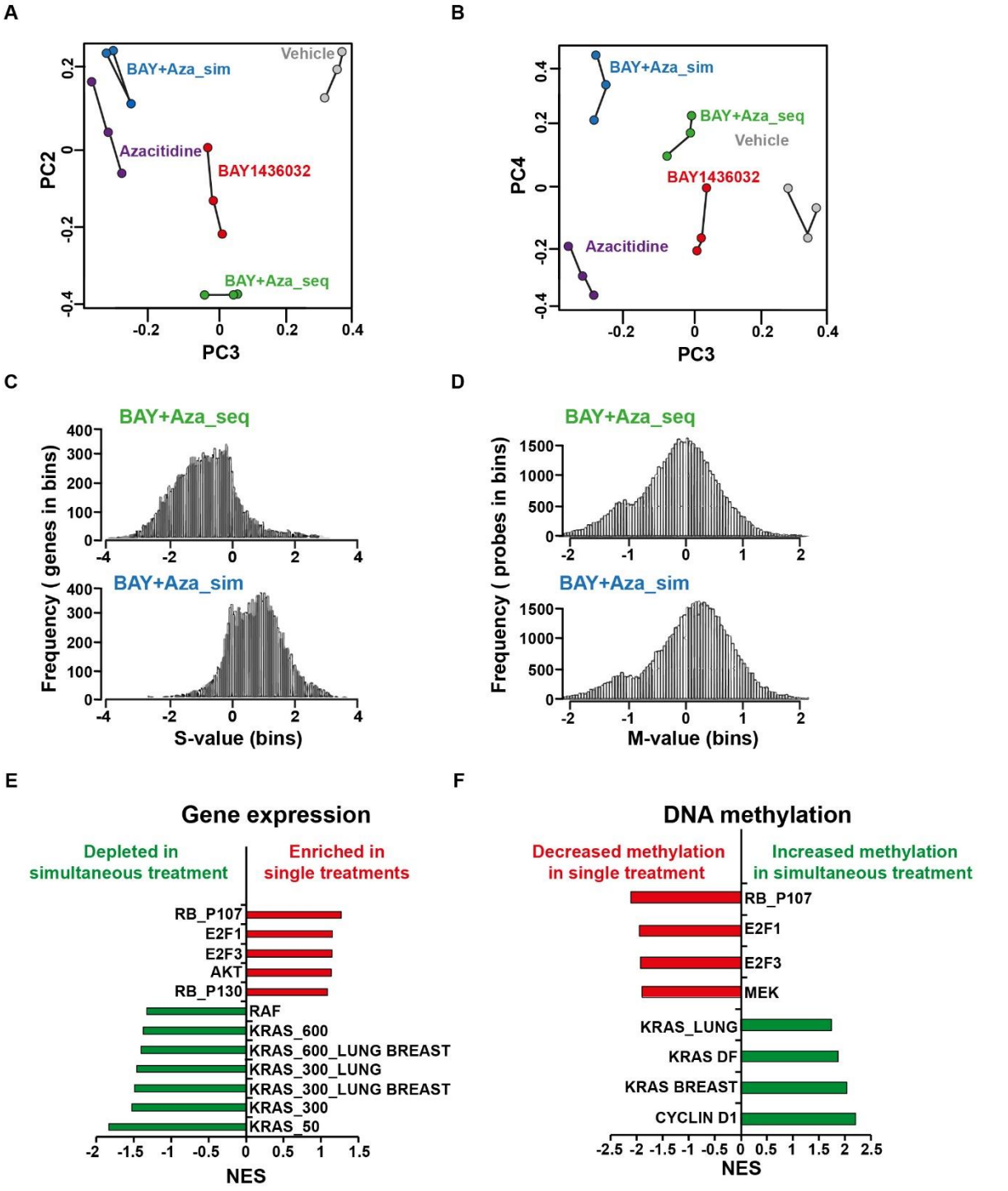
Supplementary Figure S6. Combination treatment with BAY1436032 and azacitidine depletes leukemia stem cells. (A) A representative FACS plot from the peripheral blood of mice before the start of treatment of mice used for the limiting dilution transplantation experiment, which is used to estimate the leukemia stem cell frequency. (B) The estimated leukemia stem cell frequency calculated by limiting dilution transplantation from the bone marrow of mice after treatment with either vehicle, azacitidine, BAY1436032, or the sequential or simultaneous combination of BAY1436032 and azacitidine for four weeks. The bar graph is shown in Figure 3A.

Figure S7



Supplementary Figure S7. Unsupervised hierarchical clustering of genes and cells from bone marrow of *IDH1* mutant PDX1 mice treated with vehicle, azacitidine (1 mg/kg, s.c., days 1 to 5), BAY1436032 (150 mg/kg, p.o., q.d., 4 weeks) or the simultaneous combination of BAY 1436032 and azacitidine, based on 89 genes that are differentially expressed between LSC+ and LSC- cell fractions from AML patients.[7] The genes of the 17-gene LSC score (LSC17) are marked by a star, the genes of the LSC3 score (a subset of LSC17) by 2 stars.

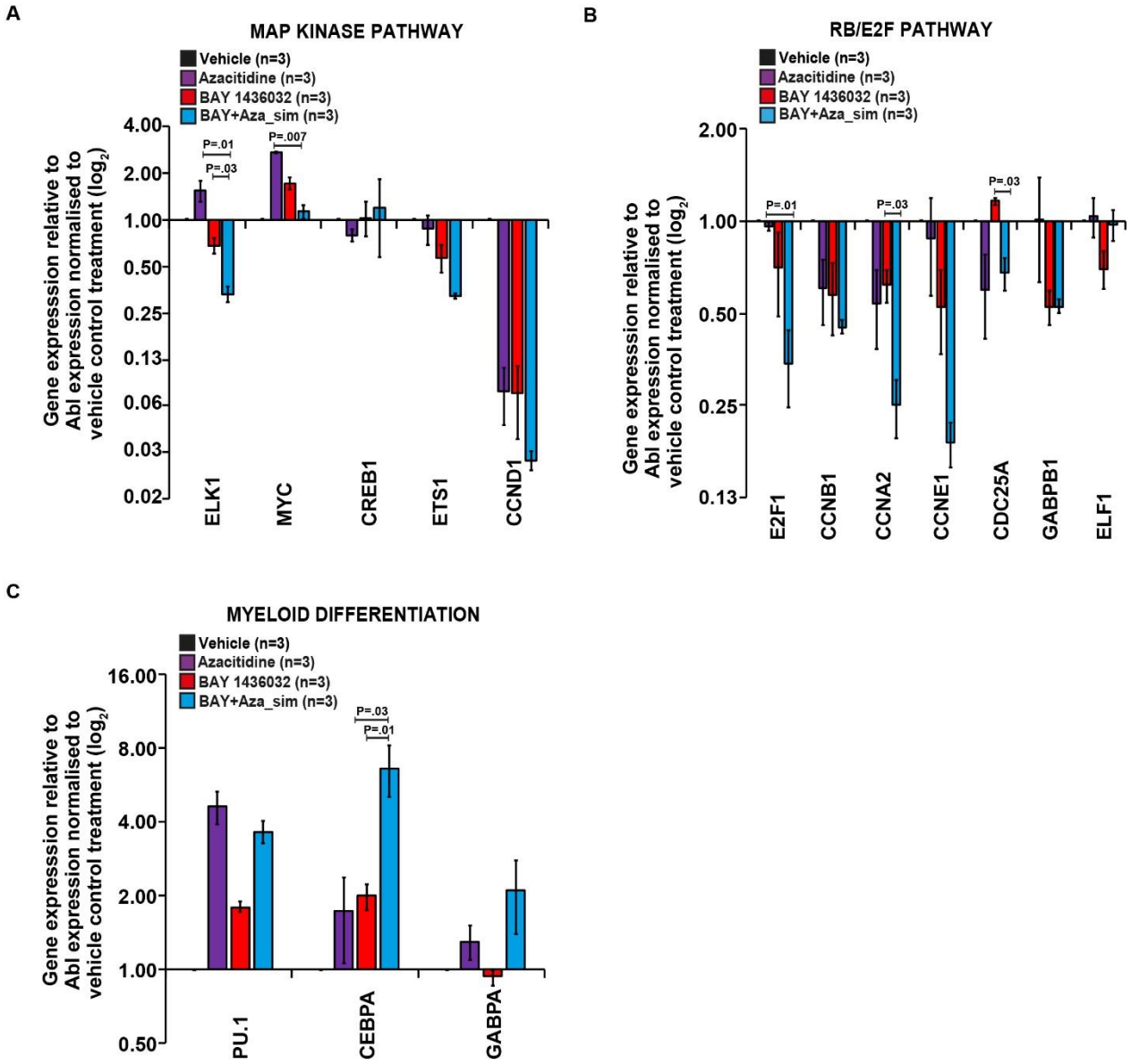
Figure S8


A
Vehicle
BAY+Aza_sim
Azacitidine
BAY1436032
BAY+Aza_seq
PC2
PC3
B
BAY+Aza_sim
BAY+Aza_seq
Vehicle
BAY1436032
Azacitidine
PC4
PC3
C
BAY+Aza_seq
BAY+Aza_sim
Frequency (genes in bins)
S-value (bins)
D
BAY+Aza_seq
BAY+Aza_sim
Frequency (probes in bins)
M-value (bins)
E
Gene expression
Depleted in simultaneous treatment
Enriched in single treatments
RB_P107
E2F1
E2F3
AKT
RB_P130
RAF
KRAS_600
KRAS_600_LUNG BREAST
KRAS_300_LUNG
KRAS_300_LUNG BREAST
KRAS_300
KRAS_50
NES
F
DNA methylation
Decreased methylation in single treatment
Increased methylation in simultaneous treatment
RB_P107
E2F1
E2F3
MEK
KRAS_LUNG
KRAS DF
KRAS BREAST
CYCLIN D1
NES

Supplementary Figure S8. Comparison of gene expression between sequential and simultaneous treatment groups and correlation of gene expression changes with changes in DNA methylation.

(A) Results of principal component analysis of all treatment groups using principal components 2 and 3. Read counts from RNASeq analysis were converted to Counts Per Million mapped reads. (B) Results of principal component analysis of all treatment groups using principal components 3 and 4 as above. (C) Distribution of gene expression synergy S-values, S=AB-A-B, where AB, A, and B are expression changes under combined (sequential or simultaneous, AB) or separate treatments [azacitidine (A) or BAY1436032 (B)]. (D) Distribution of methylation levels of Illumina MethylationEPIC probes in sequential (top) and simultaneous (bottom) treatment groups. Average_beta (B) values were converted to M-values as M=log2(B/(1-B). The top 50.000 most differentially methylated probes were selected, and normalized M-values were used to prepare the histogram. (E) Gene set enrichment analysis (MSigDB version 6.0) showing the most enriched transcription factor target gene sets from RNASeq analyses, and (F) gene set enrichment analysis (MSigDB version 6.0) showing the most enriched transcription factor target gene sets from DNA methylation analysis in primary human IDH1 mutant AML cells that were isolated from the PDX1 mouse. Simultaneous treatment refers to the simultaneous treatment of the cells with BAY1436032 and azacitidine in vitro. Single treatment refers to the treatment of the cells with either BAY1436032 alone or azacitidine alone in vitro. The two single treated groups were combined to one group and compared to the simultaneously treated group.

Figure S9



Supplementary Figure S9. Validation of gene expression changes in BAY1436032 and azacitidine treated cells by qRT-PCR. Gene expression levels of MAP kinase signaling genes (A), RB/E2F signaling genes (B) and myeloid differentiation genes (C) from the bone marrow of IDH1 mutant PDX1 mice treated with vehicle, azacitidine (1 mg/kg, s.c., days 1 to 5), BAY1436032 (150 mg/kg, p.o., q.d., 4 weeks) or simultaneous combination of BAY1436032 and azacitidine. Gene expression was determined by quantitative RT-PCR relative to the housekeeping gene ABL and was normalized to gene expression in vehicle-treated mice/cells (mean ± SEM, n= three independent experiments).

Supplementary References

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Supplementary Tables

Supplementary Table S1: Top 50 gene sets from transcription factor collection depleted in azacitidine treated cells compared to vehicle.

Transcription factors depleted in azacitidine treated cells compared to vehicle			
Rank	**NAME**	**NES**	**P**
1	HMX1_01	-1.5813	0.0000
2	AHR_Q5	-1.4931	0.0000
3	ZIC3_01	-1.4407	0.0000
4	SREBP1_Q6	-1.4345	0.0000
5	CREB_02	-1.4230	0.0000
6	HEN1_01	-1.3367	0.0000
7	SMAD_Q6	-1.3367	0.0000
8	NFMUE1_Q6	-1.3331	0.0000
9	HEN1_02	-1.3288	0.0000
10	MAZR_01	-1.3269	0.0000
11	SREBP1_02	-1.3043	0.1026
12	MYB_Q3	-1.2937	0.0000
13	CP2_02	-1.2859	0.0000
14	E2F_Q4_01	-1.2723	0.0808
15	AREB6_03	-1.2616	0.0000
16	VDR_Q3	-1.2542	0.0000
17	AP1FJ_Q2	-1.2534	0.0000
18	FAC1_01	-1.2531	0.0000
19	E2F1_Q6_01	-1.2501	0.0000
20	MZF1_02	-1.2459	0.0000
21	CREBP1_Q2	-1.2423	0.1882
22	SPZ1_01	-1.2332	0.0000
23	ZF5_B	-1.2319	0.0889
24	PAX4_01	-1.2288	0.0832
25	PAX4_03	-1.2256	0.0000
26	ETS_Q4	-1.2241	0.1149
27	SP1_Q6	-1.2239	0.1075
28	SP3_Q3	-1.2215	0.0000
29	STAT3_01	-1.2197	0.2122
30	E2F1_Q3_01	-1.2182	0.0848
31	PPAR_DR1_Q2	-1.2177	0.0000
32	EGR_Q6	-1.2170	0.2024
33	CEBPB_01	-1.2151	0.0000
34	STAT5A_02	-1.2123	0.0000
35	AP2REP_01	-1.2072	0.0873
36	CEBPDELTA_Q6	-1.2066	0.0000

37	CREB_Q2_01	-1.2037	0.0000
38	E2F4DP1_01	-1.2033	0.0000
39	CREB_Q4	-1.2030	0.0851
40	LFA1_Q6	-1.2020	0.0000
41	ETS1_B	-1.1995	0.0000
42	ATF4_Q2	-1.1978	0.0861
43	DR1_Q3	-1.1927	0.0000
44	CMYB_01	-1.1894	0.0000
45	TEL2_Q6	-1.1855	0.1191
46	PXR_Q2	-1.1853	0.0000
47	PPARA_02	-1.1818	0.1071
48	ZID_01	-1.1754	0.0000
49	AREB6_01	-1.1732	0.1394
50	MYB_Q5_01	-1.1728	0.2162

Supplementary Table S2: Top 50 gene sets from transcription factor collection depleted in BAY1436032 compared to vehicle.

Transcription factors depleted in BAY1436032 treated cells compared to vehicle			
Rank	**NAME**	**NES**	**P**
1	E2F1_Q6	-1.5809	0.0000
2	E2F_Q4	-1.5759	0.0000
3	E2F4DP1_01	-1.5494	0.0000
4	E2F1DP2_01	-1.5459	0.0000
5	E2F1DP1RB_01	-1.5154	0.0000
6	PPARG_01	-1.5117	0.0000
7	MAX_01	-1.4863	0.0000
8	MYCMAX_03	-1.4522	0.0000
9	E2F_03	-1.4455	0.0000
10	MYCMAX_B	-1.4322	0.0000
11	E2F_Q3	-1.4300	0.0000
12	NFMUE1_Q6	-1.3755	0.0000
13	USF_01	-1.3676	0.0000
14	PAX6_01	-1.3667	0.0000
15	USF_Q6	-1.3628	0.0000
16	NFY_01	-1.3468	0.0000
17	STAT1_02	-1.3412	0.0857
18	ARNT_01	-1.3389	0.0000
19	CETS1P54_01	-1.3317	0.0854
20	YY1_02	-1.3283	0.0854
21	SP1_Q6	-1.3232	0.0000
22	IRF2_01	-1.3050	0.0000
23	NRF1_Q6	-1.3045	0.0868
24	WHN_B	-1.2910	0.0000
25	NMYC_01	-1.2893	0.0000
26	HIF1_Q3	-1.2879	0.0000
27	EGR3_01	-1.2838	0.0000
28	ARNT_02	-1.2836	0.1184
29	AHR_Q5	-1.2484	0.1146
30	USF_C	-1.2003	0.1594
31	HIF1_Q5	-1.1786	0.0768
32	USF_Q6_01	-1.1696	0.2045
33	SP1_Q4_01	-1.1695	0.2004
34	CREB_Q3	-1.1639	0.1620
35	TAXCREB_01	-1.1571	0.3051
36	TEL2_Q6	-1.1558	0.2560
37	ELK1_01	-1.1525	0.1839
38	STAT3_01	-1.1369	0.2182

39	USF_02	-1.1311	> 0.0001
40	NFY_Q6	-1.1218	0.1730
41	USF2_Q6	-1.1198	0.2825
42	MYCMAX_02	-1.1053	0.2008
43	RFX1_01	-1.0893	0.2900
44	NFY_Q6_01	-1.0864	0.2769
45	NFY_C	-1.0842	0.3122
46	EGR1_01	-1.0768	0.3152
47	CREB_01	-1.0744	0.1585
48	CMYB_01	-1.0723	0.2872
49	CREB_02	-1.0673	0.2544
50	ALPHACP1_01	-1.0518	0.2004

Supplementary Table S3: Top 50 gene sets from transcription factor collection depleted in BAY1436032+azacitidine treated cells in combination compared to single agent treated cells with BAY1436032 or azacitidine or vehicle

Transcription factor collection depleted in BAY1436032+azacitidine treated cells in combination compared to single agent treated cells with BAY1436032 or azacitidine or vehicle			
Rank	**NAME**	**NES**	**P**
1	TEL2_Q6	-1.4806	0.0000
2	NRF2_01	-1.4528	0.0000
3	STAT1_02	-1.4504	0.0230
4	CETS1P54_01	-1.4393	0.0000
5	USF_C	-1.4233	0.0124
6	GABP_B	-1.4224	0.0132
7	ELK1_02	-1.4009	0.0485
8	ELK1_01	-1.4002	0.0000
9	NRF1_Q6	-1.3888	0.0106
10	XBP1_01	-1.3734	0.0000
11	ETS_Q4	-1.3423	0.0000
12	PEA3_Q6	-1.2693	0.0955
13	AR_02	-1.2662	0.1125
14	USF_Q6	-1.2348	0.0751
15	ARNT_02	-1.2179	0.1245
16	TAXCREB_01	-1.1946	0.2124
17	NFMUE1_Q6	-1.1944	0.2978
18	YY1_Q6	-1.1883	0.2018
19	USF2_Q6	-1.1865	0.1486
20	HTF_01	-1.1838	0.2541
21	USF_02	-1.1203	0.2243
22	NFY_Q6_01	-1.1196	0.2353
23	E2F1_Q6	-1.1087	0.3866
24	STAT1_03	-1.0921	0.3358
25	AHR_01	-1.0917	0.2455
26	E2F4DP1_01	-1.0893	0.4101
27	PAX2_01	-1.0801	0.2749
28	E2F1_Q6_01	-1.0727	0.4387
29	E4F1_Q6	-1.0690	0.3424
30	E2F1_Q4	-1.0657	0.4000
31	E2F1DP1RB_01	-1.0640	0.4543
32	NFY_C	-1.0613	0.3341
33	USF_01	-1.0589	0.3564
34	MYC_Q2	-1.0556	0.3535
35	NMYC_01	-1.0538	0.2346
36	PPARG_01	-1.0523	0.3776
37	E2F_02	-1.0478	0.5012

38	E2F1DP1_01	-1.0361	0.5203
39	E2F1DP2_01	-1.0361	0.5203
40	E2F4DP2_01	-1.0361	0.5203
41	E2F_Q6	-1.0358	0.4715
42	E2F_Q4	-1.0346	0.4736
43	E2F1_Q3	-1.0175	0.5201
44	E4BP4_01	-1.0135	0.4281
45	E2F_Q3	-1.0097	0.5553
46	ARNT_01	-1.0095	0.4567
47	MAX_01	-0.9983	0.3861
48	WHN_B	-0.9906	0.4354
49	ATF6_01	-0.9735	0.5466
50	MYCMAX_B	-0.9724	0.4007