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Venetoclax synergizes with Gilteritinib in FLT3 wildtype high-risk Acute Myeloid Leukemia by suppressing MCL-1

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Abstract:
BCL-2 inhibition has been shown to be effective in acute myeloid leukemia (AML) in combination with hypomethylating agents or low-dose cytarabine. However, resistance and relapse represent major clinical challenges. Thus, there is an unmet need to overcome resistance to current venetoclax-based strategies. We performed high-throughput drug screening to identify effective combination partners for venetoclax in AML. Overall, 64 anti-leukemic drugs were screened in 31 primary high-risk AML samples with or without venetoclax. Gilteritinib exhibited highest synergy with venetoclax in FLT3 wildtype AML. The combination of gilteritinib and venetoclax increased apoptosis, reduced viability, and was active in venetoclax-azacitidine resistant cell lines and primary patient samples. Proteomics revealed increased FLT3 wildtype signaling in specimens with low in-vitro response to the currently used venetoclax-azacitidine combination. Mechanistically, venetoclax with gilteritinib decreased phosphorylation of ERK and GSK3B via combined AXL and FLT3 inhibition with subsequent suppression of the antiapoptotic protein MCL-1. MCL-1 downregulation was associated with increased MCL-1 phosphorylation of serine 159, decreased phosphorylation of threonine 161 and proteasomal degradation.

Gilteritinib and venetoclax were active in a FLT3 wildtype AML PDX model with TP53 mutation and reduced leukemic burden in four FLT3 wildtype AML patients receiving venetoclax-gilteritinib off-label after developing refractory disease under venetoclax-azacitidine.

In summary, our results suggest that combined inhibition of FLT3/AXL potentiates venetoclax response in FLT3-wildtype AML by inducing MCL-1 degradation. Thus, the venetoclax-gilteritinib combination merits testing as potentially active regimen in high-risk AML patients with FLT3 wildtype.

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Key points

- High-throughput drug screening identified gilteritinib and venetoclax as a highly synergistic drug combination in FLT3 wildtype AML
- Gilteritinib-venetoclax suppressed MCL-1 and decreased viability of venetoclax-azacitidine resistant FLT3 wildtype AML in vitro and in vivo

Abstract

BCL-2 inhibition has been shown to be effective in acute myeloid leukemia (AML) in combination with hypomethylating agents or low-dose cytarabine. However, resistance and relapse represent major clinical challenges. Thus, there is an unmet need to overcome resistance to current venetoclax-based strategies. We performed high-throughput drug screening to identify effective combination partners for venetoclax in AML. Overall, 64 anti-leukemic drugs were screened in 31 primary high-risk AML samples with or without venetoclax. Gilteritinib exhibited highest synergy with venetoclax in FLT3 wildtype AML. The combination of gilteritinib and venetoclax increased apoptosis, reduced viability, and was active in venetoclax-azacitidine resistant cell lines and primary patient samples. Proteomics revealed increased FLT3 wildtype signaling in specimens with low in-vitro response to the currently used venetoclax-azacitidine combination. Mechanistically, venetoclax with gilteritinib decreased phosphorylation of ERK and GSK3B via combined AXL and FLT3 inhibition with subsequent suppression of the antiapoptotic protein MCL-1. MCL-1

downregulation was associated with increased MCL-1 phosphorylation of serine 159, decreased phosphorylation of threonine 161 and proteasomal degradation.

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In summary, our results suggest that combined inhibition of FLT3/AXL potentiates venetoclax response in FLT3 wildtype AML by inducing MCL-1 degradation. Thus, the venetoclax-gilteritinib combination merits testing as potentially active regimen in high-risk AML patients with FLT3 wildtype.

Introduction

With a median onset age of 65 years, AML is predominantly a disease of the elderly with limited intensive chemotherapy options.[1] Unfit patients are offered low-dose therapy concepts such as hypomethylating agents (HMA) or low-dose cytarabine (LDAC) associated with low remission rates and poor median survival.[2,3] Recently, the addition of the BCL-2-inhibitor venetoclax to either LDAC[4] or HMAs[5] vastly improved response rates. These findings led to Food and Drug Administration (FDA) and European Medicines Agency (EMA) approval for the combination of HMA and venetoclax in newly diagnosed patients ineligible for intensive chemotherapy. Nonetheless, a considerable fraction of AML patients does not respond and most patients relapse after initially achieving a remission upon venetoclax and azacitidine. Moreover, relapsed patients are resistant to all currently used therapies and usually die within a short time span.[5,6] The combinations used nowadays were established based on FDA-approved pre-existing low intensity therapies in AML. However, a systematic evaluation of the most effective and synergistic venetoclax combination partners is still lacking.

Venetoclax acts as a small-molecule BCL-2 homology domain 3 (BH3)-mimetic drug. Interestingly, a recent study highlighted that lymphoid cells can escape venetoclax by reprogramming energy metabolism and overexpressing MCL-1 during complex clonal shifts.[7]

In line, Jones and colleagues described shifts in metabolism in venetoclax-resistant AML.[8] Increased levels of the anti-apoptotic BCL-2-family members MCL-1 or BCL-XL were also observed in venetoclax resistant AML cells.[9,10] Moreover, it has been reported that BCL-2 is differentially expressed in subpopulations of AML cells with highest expression in malignant stem and progenitor cells and lowest expression in AML with a monocytic phenotype which express MCL-1 instead and evolved to be refractory to venetoclax.[11] However, MCL-1 inhibition carries the risk of profound toxicity to normal tissues, especially cardiac toxicity.[12] Therefore, an indirect MCL-1 targeting approach in combination with BCL-2 inhibition might be a promising therapeutic approach.

With the aim of identifying a combinatorial treatment more effective than venetoclax-azacitidine, many therapy options have been suggested in AML. These options include inhibition of PI3-kinase, CDKs, SMAC or complex I.[13,14] So far, response mechanisms are not well understood, and the most effective treatment combinations are not known.

In this study, we developed and utilized a synergism-focused drug targeting pipeline to identify the most potent venetoclax combination partners in high-risk AML.

Materials and Methods

Drug screening

Drug response assays were performed with primary AML blasts cultured in RPMI-1640 (Invitrogen, Carlsbad, California, USA) supplemented with penicillin/streptomycin (Invitrogen), L-glutamine (Invitrogen) and 10% pooled and heat-inactivated AB-type human serum (HSA) (Sigma-Aldrich, St. Louis, Missouri, USA). Cells in medium + HSA were subjected to rolling for three hours in the dark. Only samples with a viability > 90% after 3-hour prestimulation were included into the screen and added to drug precoated 384-well plates (781904, Greiner Bio One, Frickenhausen, Germany). Plates were coated with 64 drugs in five concentrations with and without venetoclax in two concentrations (1 nM, 20 nM). Cell viability was assessed after 48 hours with a Perkin Elmer EnSight using CellTiter-Glo

(G7572, Promega, Fitchburg, Massachusetts, USA) and normalized to DMSO controls, as previously described.[15]

Patient specimens

Primary AML samples were obtained from the German "Study Alliance Leukemias" (SAL) - AML Register Dresden and the "BioMaterialBank Heidelberg" (BMBH Med V). All patients provided informed consent in accordance with the Declaration of Helsinki and biobanking procedures were approved by the ethics committee of the University of Heidelberg. Mononuclear cells of AML patients were density gradient-isolated from bone marrow aspirations taken at diagnosis. Only AML patients with high-risk disease were included in the drug screen (Table S1, Fig. S1A). High-risk disease was defined either according to the ELN classification[16] risk group (n=19) or if patients were refractory after induction chemotherapy (n=12). Three primary samples were included into *in vitro* validation studies, two of them (#01 and #70) had already been included into the drug screen (Table S1).

Treatment of patients #02, #03, #05 and #70 with venetoclax-gilteritinib was performed after written informed consent on the off-label use following the principles of Helsinki.

Cell culture

HL60, MOLM13, OCI-AML 2, OCI-AML 3 and MV4-11 AML cell lines were purchased from DSMZ and cultured in RPMI1640 medium (21875091, Thermo Fisher Scientific) supplemented with 10% (HL60) or 20% (other cell lines) FBS (FBS.S 0615, Bio&SELL GmbH, Feucht, Germany). Venetoclax-azacitidine resistant HL60 cells were generated by treating cells twice weekly with increasing doses of venetoclax (S8048, Selleckchem, Houston, Texas, USA) and azacitidine (S1782, Selleckchem) for several months.

Primary human bone marrow or peripheral blood samples were obtained from AML patients who provided informed consent. Biobank procedures are approved by the "Ethikkomission Heidelberg". Mononuclear cells were density gradient-isolated and cultured as described in [17].

Colony formation Assays

250 cells (cell line) or 4000 cells (primary sample) were seeded into twelve-well plates with 550 µl methylcellulose (04230 or 04034, Stemcell technologies) supplemented with penicillin streptomycin (A2213, Biochrom GmbH) and the indicated drugs. After ten days, colonies were counted. All experiments were performed as technical triplicates and each experiment was performed at least three times.

Apoptosis Assays

Apoptosis was assessed by staining 1-5x10^5 cells per sample with Annexin V-antibody and propidium iodide (FITC Annexin V Apoptosis Detection Kit with PI, 556547, BD Biosciences) according to the manufacturer's protocol. Analysis was carried out by flow cytometry. Experiments were performed with two technical replicates and each experiment was performed at least two times.

Viability Assays

Cell viability was assessed in 96-well plates with a density of 1-5x10^4 cells per well. After 48 hours of treatment cells were stained with trypan blue (T8154, Sigma Aldrich Chem. GmbH) and viable cells were counted. Alternatively, cells were stained with MTS reagent (G3582, Promega) and analyzed on an Infinite® M1000 PRO plate reader (Tecan, Männedorf, Switzerland).

Immunoblotting

Cells were pelleted and lysed using RIPA buffer at 4°C (89900, Thermo Fisher Scientific) supplemented with protease (11836170001, Roche, Basel, Switzerland) and phosphatase inhibitor (04906845001, Roche). After centrifugation, protein concentration was determined with a Pierce BCA Protein Assay Kit (23227, ThermoFisher Scientific). Equal amounts of whole-cell lysate were mixed with 4X LDS sample buffer (NP0008, ThermoFisher Scientific)

and 10X sample reducing agent (NP0009, ThermoFisher Scientific), heated for 10 minutes at 70°C and loaded on a 4-12% tris-glycine gradient gel (XP04122BOX, ThermoFisher Scientific) for SDS PAGE. Proteins were transferred onto a nitrocellulose membrane (GE10600001, Sigma Aldrich) which was blocked with 5% BSA (T844.2, Carl Roth GmbH & Co KG) in TBST buffer. Membranes were incubated at 4°C overnight with anti-BCL-2 (1:4 000, ab692, abcam, Cambridge, UK), anti-B-ACTIN (1:5 000, A5441, Sigma Aldrich), anti-ERK (1:1 000, 4695, Cell Signaling Technology), anti-GSK-3 α/ß (1:200, sc-7291, Santa Cruz Biotechnology, Dallas, Texas, USA), anti-MCL-1 (1:4 000, ab32087, abcam), anti-pERK Thr202/Tyr204 (1, 2 000, 4370, Cell Signaling Technology), anit-pGSK-3α/ß Ser21/9 (1:1 000, 8566, Cell Signaling Technology), anti-pMCL-1 Ser159 (1:500, ab111574, abcam), anti-pMCL-1 Thr163 (1:1 000, 14765S, Cell Signaling Technology) and secondary antibodies against mouse or rabbit immunoglobulin (1:4 000, P044701-2, P044801-2, Dako/Agilent, Santa Clara, California, USA). Proteins were visualized using ECL reagent (RPN2232, GE Healthcare, Chicago, Illinois, USA) with an Amersham imager 600 (Cytiva, Chalfont St Giles, UK).

Primary AML samples were lysed using SDS lysis buffer (0,1% SDS, 50 mM Tris pH 8) supplemented with protease and phosphatase inhibitor and then processed as described above.

Overexpression and site-directed mutagenesis

Lentiviral vectors encoding for MCL-1 (140746) and corresponding empty vector (17452) were purchased from Addgene. Gene fragments for overexpression of ERK were obtained from IDT (Integrated DNA Technologies, Coralville, Iowa, USA) and corresponding empty vector from SBI (CD811A-1, SBI, Palo Alto, California, USA). Lentivirus was produced by transfecting lentiviral vectors together with psPAX2 (12260, Addgene, Watertown, Massachusetts, USA) and pMD2.G (12259, Addgene) into HEK293T cells. Virus supernatant was used to infect HL60 cells, which then underwent puromycin selection (0,7 µg/ml, P8833-10mg, Sigma Aldrich).

Site-directed mutagenesis was used to introduce the phosphorylation site mutation S159A in MCL1. The mutation was introduced by a PCR reaction (Platinum SuperFi DNA Polymerase, 12351010, ThermoFisher Scientific) with back-to-back primers (GTAGTGCCCCGTCCGTAC TGGTG and CCTCGACGCCGCCGCCAGCAG) followed by KLD treatment. Mutagenesis results were confirmed by Sanger sequencing.

PDX model

Patient derived xenograft (PDX) cells of a highly aggressive AML with complex karyotype and TP53 mutation (AML-372)[18] were injected intravenously into immunocompromised NSG mice at minimally eight weeks of age. Mice were randomly assigned to four treatment groups: gilteritinib 85 mg/kg, venetoclax 40 mg/kg, gilteritinib and venetoclax or vehicle (sterile water with 1% Tween and 3% ethanol). Treatment was started when engraftment levels of 0.2% of human CD45+ cells were reached and was conducted for 4 weeks. Bone marrow aspirations were performed four weeks upon start of treatment. Two weeks after the end of treatment, mice were sacrificed, bone marrow was isolated and analyzed by FACS for CD45 expression (clone HI30, BD Biosciences). Animal experiments were approved and performed in accordance with all regulatory guidelines of the official committee (Regierungspräsidium Karlsruhe).

Drug sensitivity and statistical analysis

All tests were performed using R version 4.0.4 and RStudio Server version 1.4.1106-5 or GraphPad Prism version 9.2.0. Data were analyzed for normal distribution before statistical analyses. Values are presented as mean ± s.d. of replicates. Two-tailed Student's *t*-test was used to determine statistical significance unless stated otherwise. For ex vivo drug screens, synergy scores were computed according to the Bliss Independence model[19] and the Zero Interaction Potency (ZIP) model[20] with the synergyfinder R-package version 2.4.15[21]. IC_{50} scores were computed automatically using this R-package, normalized for less than 70% variance and outliers. Relative inhibition (RI) scores were computed according to the area under the curve (AUC) of the viability curves. The RI scores indicate the proportion of the maximum possible inhibition of each drug. The shiny app for data sharing utilizes r-base:4.1.3 installed from docker. Previously analyzed results are presented using ggplot2 3.3.5, ComplexHeatmap 2.10.0 and corrplot 0.92. Synergy for individual samples is freshly calculated with synergyfinder 3.2.6. The graphical abstract was created with BioRender.com.

Data Sharing Statement

For original data, please contact Carsten.Mueller-Tidow@med.uni-heidelberg.de. A comprehensive visualization of results for viability, synergy and relative inhibition for all samples from the drug screen is available in a shiny app (https://shiny-portal.embl.de/shinyapps/app/07_drug-screen).

Results

High-throughput drug screening identifies venetoclax combination partners for high-risk AML

We conducted a high-throughput ex vivo drug perturbation experiment with venetoclax and drugs targeting relevant pathways in myeloid malignancies (Fig. 1A). Thirty-one high-risk AML patient samples (Table S1a, S2-4, Fig. S1A) were incubated with venetoclax (0, 1 and

20 nM) and 64 drugs in five concentrations. High-risk was defined as either refractory to conventional chemotherapy or high-risk status according to ELN2017 guidelines.[16]

To assess the reliability of our ex vivo drug screening platform, we clustered the drugs targeting various pathways (Fig. S1B) based on the similarity of their response profiles across all AML samples (Fig. S1C). The clustering reflected drug target identity and relatedness of drugs (Fig. S1D). Patients in vivo responses to cytarabine induction therapy could be reproduced in the ex vivo drug screen (Fig. S2A).

Drug synergy effects were assessed with the Bliss Independence model.[19] For validation, we applied the Zero Interaction Potency (ZIP) model.[20] The top three venetoclax combination partners identified in our screen were MIK665 (MCL-1 inhibitor), OTX015 (BET inhibitor) and gilteritinib (FLT3 inhibitor) (Fig. 1B). The MCL-1 inhibitor MIK665 showed the strongest effects in combination with venetoclax supporting growing evidence that the anti-apoptotic protein MCL-1 confers resistance to BCL-2 inhibition in AML.[22] The bromodomain inhibitor OTX015 has been described in preclinical studies to act synergistically with venetoclax by reducing MCL-1 levels.[23] Our screening approach further identified the clinically approved drug gilteritinib as a highly active combination partner for venetoclax (Fig. 1B, Fig. S2B).

We further analyzed combination effects in subgroups of patients with different mutations (Fig. S2C-E, S3A-D). As expected, there was a trend towards a higher monotherapeutic effect of gilteritinib in FLT3 mutated AML compared to FLT3 wildtype specimens (p=0.075, Fig. 1C). However, the synergistic effect of venetoclax and gilteritinib was stronger in FLT3 wildtype than in FLT3 mutated AML (p=0.0427, Fig. 1C, Fig. S2C-D, S3A-B). In line, RI and bliss scores were higher in samples with FLT3 wildtype than in samples with FLT3 ITD (Fig. S4A-B). Highest synergy was observed for gilteritinib or MIK665 combined with venetoclax when compared to standard AML treatments (S4C). The synergistic effect with venetoclax was restricted to gilteritinib and was not observed for other FLT3 inhibitors such as quizartinib, midostaurin or sorafenib (Fig. 1D, Fig. S4D). Interestingly, gilteritinib was the top synergistic combination partner for venetoclax in the subgroup of TP53 mutated patients (Fig. 1E, Fig. S4E, Table S3-S4), for whom reduced responses to venetoclax with HMAs or LDAC

have been reported.[24] Results of exemplary patients included at first diagnosis without FLT3 ITD, but with TP53 mutation showed that gilteritinib exerted high synergism with venetoclax whereas other standard therapeutic approaches did not or weakly synergize with venetoclax (Fig. 1F, Fig. S5A-E).

Taken together, our ex vivo drug perturbation approach identified gilteritinib and venetoclax as effective combination partners with high synergy in high-risk AML with FLT3 wildtype and TP53 mutation.

Proteomics profiling reveals upregulation of FLT3 signaling in venetoclax-resistant AML

Next, we aimed to understand what drives resistance to the FDA/EMA-approved venetoclax-azacitidine combination in FLT3 wildtype samples. We chose six primary FLT3 wildtype samples based on their drug screen ex vivo responses to venetoclax-azacitidine and the availability of sufficient material for FACS-sorting and mass spectrometry (Fig. 2A). Two primary samples were classified as venetoclax-azacitidine sensitive and four primary samples as venetoclax-azacitidine insensitive according to calculated synergy scores (Fig. 2B). Venetoclax-azacitidine insensitive AML samples were enriched for proteins involved in FLT3 pathway activity, e.g. RAF/MAP, FLT3, MAPK1/MAPK3 (NES for FLT3 signaling: 1.83 adj. p-value: 0.0001; Fig. 2C-D, Table S5-6). These findings suggested that high FLT3 signaling is associated with venetoclax-azacitidine resistance in FLT3 wildtype AML.

Venetoclax-gilteritinib reduces viability and colony formation capacity and induces apoptosis in FLT3 wildtype AML.

Next, we recapitulated the combinatorial effect of venetoclax-gilteritinib in FLT3 wildtype and FLT3 ITD cell lines. Ex vivo drug screens (Fig. S6A-B) reflected the result obtained in primary samples. Upon the addition of 20 nM venetoclax, we observed an increase in RI in FLT3 wildtype cell lines whereas only a minor difference was found for the FLT3 ITD samples (Fig S6C). In line, dose-response assays of venetoclax and gilteritinib in FLT3 wildtype HL60 and

OCI-AML 2 showed decreased cell viability after 48 hours in concentrations that can be reached in plasma of patients (Fig. 3A).[25] The reduced cell viability was associated with increased apoptosis (Fig. 3B-C). Further, the drug combination synergistically reduced colony formation capacity in three FLT3 wildtype AML cell lines (Fig. 3D-E, Table S7).

The venetoclax-gilteritinib combination suppresses ERK and GSK3A/B phosphorylation and induces proteasomal degradation of MCL-1

MCL-1 confers resistance to venetoclax.[22] We thus examined protein expression of MCL-1 in HL60 cells after exposure to gilteritinib, venetoclax or the combination. For gilteritinib, a concentration of 1 µM, that is slightly higher than usual drug plasma concentrations in patients,[25] was chosen for optimal visualization of drug effects. MCL-1 protein expression decreased in venetoclax-gilteritinib exposed cells compared to non-treated or single agent treated cells (Fig. 4A). Besides other kinases, gilteritinib predominantly inhibits FLT3 and AXL and thereby affects downstream signaling pathways such as PI3K/AKT and Ras/Raf/ERK. Activation of most of these pathways occurs in FLT3 wildtype and mutant cells.[26] The gilteritinib-venetoclax combination inhibited phosphorylation of ERK1/2 in FLT3 wildtype HL60 cells (Fig. 4A). MCL-1 has a short half-life and is constantly degraded by the proteasome.[27] Degradation of MCL-1 is mediated by various mechanisms which are not yet entirely understood.[28] In addition to decreasing MCL-1 levels under gilteritinib-venetoclax, we observed a decrease of GSK3A/B phosphorylation (Fig. 4B). Phosphorylation of GSK3 by pERK has been shown to reduce kinase activity.[29] We further observed that MCL-1 threonine 163 (T163) phosphorylation levels decreased upon exposure to the venetoclax-gilteritinib combination (Fig. 4C). T163 phosphorylation induced by pERK stabilizes MCL-1.[28] In addition, we found that venetoclax-gilteritinib increased MCL-1 serine 159 (S159) phosphorylation whereas the single drugs did not (Fig. 4C). Additional phosphorylation at S159 has been shown to be mediated by GSK3A/B and increased MCL-1 proteasomal degradation.[29] In line, decreased pT163 and increased pS159 levels were associated with reduced levels of total MCL-1 in HL60 treated with venetoclax and gilteritinib (Fig. 4C) and

suggested proteasomal degradation of MCL-1. We performed venetoclax, gilteritinib and combination treatment of HL60 cells in the presence and absence of the proteasome inhibitors carfilzomib and ixazomib, respectively. MCL-1 levels increased upon proteasome inhibition in cells treated with gilteritinib mono or the drug combination (Fig. 4D, Fig. S7A). To evaluate whether the inactivation (i.e. dephosphorylation) of ERK by gilteritinib-venetoclax is important for drug efficiency, we treated HL60 cells with venetoclax, gilteritinib, venetoclax-gilteritinib or the combination of the ERK inhibitor SCH772984 with venetoclax. As venetoclax-gilteritinib, the combination of ERK inhibition with venetoclax reduced HL60 viability (Fig. S7B). Immunoblotting of treated cells further demonstrated that direct ERK inhibition in combination with venetoclax strongly decreased pMCL-1 T163 and thus reduced total MCL-1 levels comparable to venetoclax-gilteritinib (Fig. S7C). After overexpressing ERK, levels of pMCL-1 T163 increased (Fig. S7D), and total MCL-1 levels remained stable upon treatment with venetoclax-gilteritinib (Fig. S7E). Hence, reduction of pERK mediated phosphorylation of MCL1 at T163 is crucial for the mode of action of venetoclax-gilteritinib. The inhibition of GSK3, on the other hand, reduced the combination effect of gilteritinib-venetoclax and partially prevented the downregulation of MCL-1 (Fig. S7F-G).

Taken together, the combination of venetoclax and gilteritinib reduced levels of pERK, pGSK3A/B and pMCL1 T163 and induced S159 phosphorylation, which was associated with proteasomal degradation of MCL-1.

MCL-1 S159A mutant cells do not respond to venetoclax-gilteritinib

We sought to confirm the crucial role of S159 phosphorylation for efficacy of venetoclax-gilteritinib by lentiviral overexpression of MCL-1 with a phosphorylation-resistant S159A mutation (Fig. 5A). MCL-1 S159A overexpressing cells showed increased resistance towards venetoclax (Fig. 5B). The venetoclax-gilteritinib combination was not synergistic and the MCL-1 S159A cells were resistant to the drug combination (Fig. 5C-D), underlining the importance of MCL-1 degradation for the effect of venetoclax-gilteritinib.

Dual inhibition of FLT3 and AXL is essential for venetoclax and gilteritinib synergism

Gilteritinib affects kinases beyond FLT3, including the AXL receptor tyrosine kinase. We utilized bemcentinib, a specific AXL inhibitor to analyze involvement of AXL in the synergistic effects of venetoclax-gilteritinib. MCL-1, pERK and pGSK3 levels were following bemcentinib-venetoclax treatment (Fig. 5E). In contrast to gilteritinib, the FLT3 inhibitors quizartinib and midostaurin, which do not target AXL, did not reduce levels of MCL-1, pERK and pGSK3 in combination with venetoclax (Fig. S7H-I). In line, treatment with quizartinib alone or in combination with venetoclax did not reduce viability of HL60 wildtype cells as did gilteritinib with or without venetoclax (Fig. 5F). However, bemcentinib added to venetoclax-quizartinib mimicked the effects of gilteritinib-venetoclax (Fig. 5F). These findings suggested that dual targeting of FLT3 and AXL was required for synergistic effects of gilteritinib and venetoclax.

Gilteritinib combined with venetoclax reduced engraftment of a FLT3 wildtype, TP53 mutated PDX model in vivo

Next, we injected NSG mice with a highly aggressive PDX model (AML 372) to analyze in vivo efficiency of venetoclax-gilteritinib (Table S8). A FLT3 wildtype model with TP53 mutation was chosen because the drug screen suggested highest activity in AML with a TP53 mutation. Mice were divided into four treatment groups: venetoclax, gilteritinib, venetoclax-gilteritinib and vehicle (Fig. 6A). Within the fourth week of treatment, engraftment was analyzed. Lowest engraftment was observed in the venetoclax-gilteritinib group, which was significantly different from engraftment levels in the control group (Fig. 6B). Two weeks post-treatment, mice were sacrificed. Again, percentage of CD45+ blasts was lowest in the venetoclax-gilteritinib group. Interestingly, only engraftment levels within the venetoclax-gilteritinib group remained significantly lower compared to the vehicle group ($p=0.0308$, Fig. 6B). If only samples with a bone marrow engraftment > 0.3% were included, engraftment

within the venetoclax-gilteritinib group was significantly reduced compared to the venetoclax group (p=0.0326, Fig. S8A).

In conclusion, the venetoclax-gilteritinib combination is capable of effectively reducing FLT3 wildtype, TP53 mutated AML .

Venetoclax-azacitidine relapsed/refractory AML patients respond to gilteritinib and venetoclax

Patients relapsing after venetoclax-azacitidine treatment do not respond to any known treatment.[30] We treated four relapsed or refractory FLT3 wildtype AML patients for whom further treatment options had been exhausted with venetoclax and gilteritinib in off-label use. All patients had previously undergone allogeneic stem cell transplantation. Two of them (#02 and #70) relapsed following treatment with venetoclax-azacitidine. One patient (#03) was upfront refractory to venetoclax and azacitidine as well as to a treatment combination including high-dose cytarabine following the second relapse. For patient #02, treatment with venetoclax and gilteritinib led to a rise in absolute neutrophil count (Fig. 6C), peripheral blast clearance and a bone marrow blast reduction from 53 to 30% (Fig.6D). Unfortunately, this patient suffered from infectious complications with no further blast reduction and died four weeks later. For patient #70, treatment with venetoclax and gilteritinib led to a peripheral blast reduction (Fig. 6E) and a rise in absolute neutrophil counts (ANC) above 1.0/nl (Fig.6C). In patient #03, the combination approach led to a reduction in bone marrow blasts from 70 to 40% (Fig.6F). However, pancytopenia due to heavy pretreatment could not be resolved. Since the blast count was still at 40% eight weeks after the start of venetoclax-gilteritinib treatment, this palliative treatment approach was stopped. Patient #05 relapsed following 15 courses of venetoclax-azacitidine. Bone marrow aspiration displayed blast counts of 8%, while the NPM1 level rose to 286%. Following three weeks of treatment with venetoclax-gilteritinib, bone marrow aspiration displayed cytologic CRi and NPM1 levels dropped to 81% (Fig. 6G-H). The treatment was discontinued because of neutropenia CTCAE grade 3 and

infectious complications. The treatment was not reinitiated based on patient decision and changed to best supportive care.

We analyzed the expression of MCL-1 in bulk bone marrow blasts obtained from patient #02 and #70 under venetoclax-azacitidine and venetoclax-gilteritinib treatment, respectively. Notably, MCL-1 was strongly depleted in blasts from patient #70 and slightly decreased in blasts from patient #02 under venetoclax-gilteritinib (Fig. 6I).

To replicate the venetoclax-gilteritinib treatment findings *in-vitro*, we treated blasts from patients #02 and #70 with venetoclax and gilteritinib in cell culture. Samples from patient #70 were also included in our *ex vivo* drug screen (Table S1), and here venetoclax-gilteritinib had additive effects whereas response to venetoclax and azacitidine was weak (Fig. S8B). No material for *in vitro* analysis was available from patient #03, #04 and #05. Thus, we included material from an additional FLT3 wildtype patient (#01) with a second relapse 20 months after allogeneic stem cell transplantation at progressive disease who was upfront refractory to venetoclax and azacitidine (Table S1).

In vitro, the venetoclax-gilteritinib combination reduced cell viability synergistically (Fig. S8C), whereas venetoclax and azacitidine did not (Fig. S8D). Further, the combination significantly reduced colony formation capacity in a synergistic manner (Fig. S8E). MCL-1 protein levels were suppressed upon venetoclax and gilteritinib treatment in bone marrow blasts from patient #01 (Fig. 6J) from whom sufficient material was available for western blotting.

Venetoclax-azacitidine resistance correlates with upregulation of MCL-1 and FLT3-downstream pathways

We generated resistant HL60 cells (HL60R) by constant exposure to increasing concentrations of venetoclax and azacitidine (Fig. 7A). HL60R cells expressed high levels of MCL-1 (Fig. 7B). Nonetheless, gilteritinib and venetoclax-gilteritinib decreased cell viability and inhibited colony formation capacity of HL60R (Fig. 7C-D). Proteome analyses of parental HL60 and HL60R cells revealed that several FLT3 downstream signaling pathways were

upregulated in venetoclax-azacitidine resistant cells. FLT3 as well as MAPK signaling associated proteins were enriched in HL60R (Fig. 7E-F, Table S9-S10).

Discussion

Venetoclax combination therapy constitutes a major breakthrough in AML. However, relapses occur frequently and few if any high-risk AML patients are cured by the currently approved venetoclax combinations. Identification of the best synergistic combinations might improve patients' responses and remission duration. But these efforts are hampered by the fact that venetoclax is rather ineffective as monotherapy in AML. In addition, synergism drug screens with primary cancer cells are challenging.

In our high-throughput drug screening with primary high-risk AML specimens, gilteritinib was identified as a promising combination partner for venetoclax therapy in FLT3 wildtype specimens. Synergistic activity of venetoclax and gilteritinib has already been reported in FLT3 mutated AML in vitro and in vivo.[31-35] Prior *in vitro* studies also detected synergistic effects of venetoclax and gilteritinib in FLT3 wildtype samples, but these initial findings were not further investigated[31,32] and the mechanism of action is unknown. An anti-leukemic effect of gilteritinib in FLT3 wildtype AML patients was also observed in clinical studies.[35] This effect might be explained by an autocrine activation of the non-mutated FLT3 kinase in AML patients.[36] The high synergism with venetoclax is specific for gilteritinib and was not found for other FLT3 inhibitors like midostaurin or quizartinib in our drug screen. Gilteritinib also inhibits AXL, a kinase that is significantly upregulated in AML samples.[37,38] In our study, effects of either AXL inhibition with venetoclax or FLT3 inhibition (quizartinib, midostaurin) with venetoclax were lower than venetoclax-gilteritinib effects, respectively. Accordingly, combined AXL and FLT3 targeting is the likely mechanism for gilteritinib-venetoclax synergism in FLT3 wildtype AML.

Induction of MCL-1 was identified as a major mechanism of resistance against therapy with the BCL-2 inhibitor venetoclax.[22] FLT3 downstream signaling was found to induce

upregulation of MCL-1.[39,40] AXL inhibition has been demonstrated to reduce MCL-1 levels in CLL samples.[41] Both receptor tyrosine kinases, FLT3 and AXL, support proliferation and survival of AML cells through PI3K/AKT, Ras/Ref/MEK/ERK, and JAK/STAT signaling pathways.[42] We identified ERK inhibition with subsequent activation of GSK3A/B, increased pS159 and decreased pT163 leading to reduced MCL-1 levels as mechanism of action of the gilteritinib-venetoclax combination. In our experiments, higher concentrations of gilteritinib even reestablished venetoclax sensitivity in MCL-1 overexpressing cells.

MCL-1 is known to be regulated by various pathways.[28] AML clones selected during venetoclax and azacitidine treatment are described to be more monocytic with higher expression of MCL-1[11]. A reasonable treatment approach could be to add gilteritinib early to venetoclax in order to abolish formation of resistant MCL-1 expressing clones.

Overall, gilteritinib-venetoclax was synergistic in FLT3 wildtype primary high-risk specimens. Of note, the combination reduced blast counts in several heavily pretreated FLT3 wildtype patients who lacked other treatment options. Given the late-stage disease of the patients and the individualized concepts, no further conclusions can be drawn at this time. A clinical trial is required to assess the venetoclax-gilteritinib combination in FLT3 wildtype AML patients.

Taken together, our study shows that rational in vitro drug testing opens new avenues to further improve venetoclax-based treatment options in AML.

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Declaration of Interests

The authors declare no competing financial interest.

Author contributions

MJ designed the study, performed experiments and analyses and wrote the manuscript; CS performed experiments and analyses and wrote the manuscript; PMB performed experiments and analyses and edited the manuscript; MFB performed proteomics experiments and analyses; CR performed computational analyses; AW performed experiments and analyses and edited the manuscript; DH performed experiments and analyses; SG performed analyses and wrote the manuscript; SR performed experiments and analyses and edited the manuscript; SAH performed experiments; MK performed experiments; CK performed experiments; LV performed experiments; BB performed experiments; AP performed experiments; KW performed experiments; AKL performed experiments; MF performed experiments and analyses; MG performed experiments and computational analyses; RFS edited the manuscript; FS edited the manuscript; MB edited the manuscript; CR edited the manuscript; UP edited the manuscript; CB edited the manuscript; HS edited the manuscript; TS edited the manuscript; SR edited the manuscript; CP co-supervised the project and edited the manuscript; GV supervised experiments and data analyses; BV performed experiments and analyses, co-supervised the project and edited the manuscript; IJ co-supervised the project and edited the manuscript; AT co-supervised the project and edited the manuscript; JK co-supervised the project and edited the manuscript; CMT designed the study, supervised

the project and wrote the manuscript; SD designed the study, supervised the project and wrote the manuscript.

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Figure legend

Figure 1 – High-throughput drug screening approach identified gilteritinib as synergistic combination partner for venetoclax.

(A) Experimental setup of the drug screening approach. 31 high-risk AML patient samples were treated with venetoclax (0, 1, 20 nM) with 64 different drugs in five different concentrations for 48 hours. Maximum concentrations used in the drug screen were IC_{50} concentrations found in literature, all other concentrations were deduced from division steps by five. Viability was determined as a readout using CellTiter-Glo, and synergism scores (bliss, ZIP) were calculated using the synergyfinder R-package version 2.4.13[18]. (B) Waterfall plot of mean bliss scores of all drugs combined with venetoclax (calculated as the mean over all bliss scores reached with each drug in five concentrations combined with venetoclax in two concentrations). Bliss synergy score was calculated as described in [16]. Colours indicate targets of the respective drugs. Waterfall plot is shown for all primary AML samples (n=31). (C) Relative inhibition (RI) reached by gilteritinib mono (left) and maximum bliss synergy scores for gilteritinib and venetoclax reached in all tested concentrations (right) in FLT3 mutated (n=10) or FLT3 wildtype (n=17) samples. RI scores were computed according to the area under the curve (AUC) of the viability curves. The RI scores indicate the proportion of the maximum possible inhibition of each drug independent of a single concentration. Mean RI

and mean maximum bliss, respectively, of individual patient samples are shown. Colours indicate the FLT3 mutational status. Statistical significance was assessed using a two-tailed Student's unpaired t-test. *p≤0.05. (D) Heatmap depicting bliss scores for venetoclax combined with different FLT3 inhibitors in FLT3 wildtype patient samples. (E) Waterfall plot of mean bliss scores of all drugs combined with venetoclax (calculated as the mean over all bliss scores reached with each drug in five concentrations combined with venetoclax in two concentrations). Bliss synergy score was calculated as described in [16]. Colours indicate targets of the respective drugs. Waterfall plots is shown for the subgroup of patients with TP53 mutations obtained at first diagnosis (n=6). (F) Bliss synergy scores of venetoclax in combination with gilteritinib, azacitidine, cytarabine and daunorubicin, respectively, in a patient sample with TP53 mutation and FLT3 wildtype. Colours indicate synergism calculated as described in [16]. Synergy scores of ≥ 0 are regarded as synergistic.

Figure 2 – Proteomics of primary AML patient samples revealed upregulation of FLT3 and MAPK signaling in venetoclax-azacitidine resistant samples.

(A) Experimental setup of the proteomics experiments conducted with primary patient samples. FLT3 wildtype samples (n=6) were divided into two groups according to bliss scores achieved by venetoclax and azacitidine in the drug screening approach (high-responders (n= 2) vs. low-responders (n= 4)). Cells were sorted for high CD34 and moderate CD45 expression, and whole proteome was examined by mass spectrometry and compared. (B) Maximum venetoclax-azacitidine bliss scores of FLT3 wildtype patient samples analyzed by proteomics. A bliss > 5 was defined as high response. (C) NES plot for FLT3 (left) and MAPK (right) signaling in AML samples with high ex vivo response vs. low response to venetoclax-azacitidine. (D) Heatmap of FLT3 signaling associated (top) and MAPK signaling associated (bottom) proteins differentially expressed in AML samples with high ex vivo response vs. low response to venetoclax-azacitidine.

Figure 3 – Combination of gilteritinib and venetoclax reduced viability, increased apoptosis, and diminished colony formation capacity in FLT3 wildtype samples.

(A) Dose response assay for HL60 (left) and OCI-AML 2 (right) cells treated with indicated concentrations of venetoclax and gilteritinib for 48 hours. Viability was assessed by staining with MTS reagent and was normalized to untreated controls. Data are presented as mean ± s.d. of three technical replicates of one representative independent experiment (n=3). (B) Percentage of apoptotic cells was measured by flow cytometry in HL60 and OCI-AML 2 cells 24 hours after treatment with venetoclax (0, 10, 100 nM) and gilteritinib (0, 500 nM) upon staining with Annexin V-antibody and PI. Data are presented as mean ± s.d. of two independent experiments comprising two technical replicates each. Statistical significance was assessed using a two-tailed Student's unpaired t-test. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$. Bliss scores are given in a range of -100 to 100 with 100 as maximum bliss score. (C) Representative images of FACS analysis of OCI-AML 2 following 24 hours treatment with gilteritinib (0, 500 nM) and venetoclax (10, 100 nM). (D) Effect of venetoclax and gilteritinib onto colony formation capacity of HL60, OCI-AML 2 and OCI-AML 3 cells was assessed by seeding cells in methylcellulose supplemented with the respective drugs for ten days. Data of three independent experiments with three technical replicates each are presented. Bliss scores are given in a range of -100 to 100 with 100 as maximum bliss score. (E) Representative microscopy images of colony formation assays using HL60 cells treated with indicated concentrations of gilteritinib and venetoclax for ten days.

Figure 4 – Venetoclax and gilteritinib reduced ERK and GSK3B phosphorylation and MCL-1 protein levels via proteasomal degradation.

(A) Protein expression of MCL-1, BCL-2, pERK and total ERK in HL60 cells treated for indicated time span with 1 µM gilteritinib, 20 nM venetoclax or the combination of both as analyzed by western blotting. B-ACTIN levels are given as loading control. All western blot images have been cropped for improved clarity and conciseness. Quantification was

performed using ImageJ. Data are representative for three independent experiments. (B) Protein expression of pGSK3A and B, total GSK3A and B and MCL-1 in HL60 cells treated for indicated time span with 1 µM gilteritinib, 20 nM venetoclax or the combination of both as analyzed by western blotting. VINCULIN levels are given as loading control. Quantification was performed using ImageJ. Data are representative for three independent experiments. (C) Protein expression of MCL-1, pMCL-1 S159 and pMCL-1 T163 in HL60 cells treated for indicated time points with 1 µM gilteritinib, 20 nM venetoclax or the combination of both as analyzed by western blotting. B-ACTIN levels are given as loading control. Quantification was performed using ImageJ. Data was obtained from the same biological replicate as data shown in (A) and is representative for three independent experiments. (D) Protein expression of MCL-1, BCL-2, pERK and total ERK in HL60 cells treated with 1 µM gilteritinib, 20 nM venetoclax or the combination of both with or without addition of the proteasome inhibitor carfilzomib for 4 hours. B-ACTIN levels are given as loading control. Quantification was performed using ImageJ. Data are representative for three independent experiments.

Figure 5 – Overexpression of MCL-1 with S159A mutation induced resistance to venetoclax and co-targeting of FLT3 and AXL kinase is crucial for combination effect

(A) Protein expression of MCL-1 in parental HL60, empty vector transduced, MCL-1 overexpressing and MCL-1 S159A mutated HL60 cells as analyzed by western blotting. B-ACTIN levels are given as loading control. Data are representative for three independent experiments. (B) IC_{50}s for venetoclax, azacitidine and gilteritinib in empty vector transduced, MCL-1 overexpressing and MCL-1 S159A mutated HL60 cells were measured by treating cells in triplicates with the drugs in seven concentrations (1 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 µM, 10 µM) for 48 hours and staining with MTS reagent. IC_{50} was calculated at grcalculator.org and representative results of three independent experiments are shown. (C) Effect of 20 nM venetoclax, 500 nM gilteritinib or the combination of both onto viability of

empty vector transduced, MCL-1 overexpressing and MCL-1 S159A mutated HL60 cells. Viability was assessed by staining with MTS reagent and normalized to untreated cells. Data are presented as mean ± s.d. of three technical replicates of one representative independent experiment (n=3). (D) Venetoclax-gilteritinib bliss scores calculated from effect of the drug combination on viability of respectively transduced HL60 cells. Bliss scores are given in a range of -100 to 100 with 100 as maximum bliss score. Bliss scores of ≥0 indicate synergy. (E) Protein expression of MCL-1, pERK, ERK, pGSK3 and GSK3 in HL60 cells treated for indicated time span with 1 µM bemcentinib, 20 nM venetoclax or the combination of both as analyzed by western blotting. B-ACTIN levels are given as loading control. Quantification was performed using ImageJ. Data are representative for three independent experiments. (F) Effect of 20 nM venetoclax, 1 µM gilteritinib, 1 µM bemcentinib, 1 µM quizartinib or various combinations of the drugs onto viability of HL60 cells. Viability was assessed by staining with MTS reagent and normalized to untreated cells. Data are presented as mean ± s.d. of three technical replicates of one representative independent experiment (n=3).

Figure 6 – Primary AML from patients with venetoclax-azacitidine-refractory disease respond to venetoclax and gilteritinib in vitro and in vivo.

(A) Overview on PDX model experiment: 20 mice were injected with PDX cells. Treatment with gilteritinib, venetoclax, combination of both or vehicle started three weeks post injection and lasted four weeks. One animal of the control group died a few days after the injection of AML cells before the treatment was started and was considered as drop out. Two weeks after treatment stop, mice were sacrificed and bone marrow was analyzed. (B) Percentage of CD45+ cells in bone marrow obtained from PDX transformed mice in the fourth week of treatment (left) or after sacrifice (right). *$p<0.05$. (C) Absolute neutrophile count for patients #2 and #70 upon treatment with venetoclax-gilteritinib. (D-G) Percentage of bone marrow (D, F, G) or peripheral blood (E) blasts of four venetoclax-azacitidine refractory patients treated with venetoclax-gilteritinib. Blast percentage was analyzed at indicated time points upon start of the respective treatment condition. (H) Percentage of NPM1 level of patient#05. NPM1

level was analyzed at indicated time points upon start of the respective treatment condition. (I) Blasts from patients #02 and #70 obtained at different stages of therapy were lysed and analyzed for levels of MCL-1 and B-ACTIN. (J) Primary AML blasts from patient #01 were treated for twelve hours with 100 nM venetoclax, 500 nM gilteritinib or the combination of both. MCL-1 and B-ACTIN levels were detected by western blotting and compared to untreated cells.

Figure 7 – Venetoclax-azacitidine resistance is associated with upregulation of MCL-1 and FLT3 signaling and could be partly overcome by gilteritinib.

(A) IC_{50}s for venetoclax, azacitidine and gilteritinib in sensitive and venetoclax-azacitidine resistant HL60 were measured by treating cells in triplicates with the drugs in seven concentrations (1 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 µM, 10 µM) for 48 hours and staining with MTS reagent. IC_{50} was calculated at grcalculator.org and representative results of three independent experiments are shown. (B) MCL-1 levels in sensitive and resistant HL60 as estimated by western blotting. Blot is representative for three independent experiments. (C) Resistant HL60 were treated in technical triplicates with venetoclax (0, 1, 10, 20, 50, 100, 500 nM) and gilteritinib (0, 100, 500, 1 000 nM) for 48 hours. Viability was assessed by staining with MTS reagent. Data are presented as mean ± s.d. from one of three independent experiments. (D) Effect of venetoclax-gilteritinib combination on colony formation capacity of resistant HL60 was assessed by seeding cells in methylcellulose supplemented with the respective drugs for ten days. Data of three independent experiments with three technical replicates each are shown. Bliss scores are given in a range of -100 to 100 with 100 as maximum bliss score. (E) NES plot for FLT3 (left) and MAPK (right) signaling in sensitive vs. venetoclax-azacitidine resistant HL60 cells. (F) Heatmap of MAPK (left) and FLT3 (right) signaling associated proteins differentially expressed in sensitive vs. venetoclax-azacitidine resistant HL60 cells.