MDM2- and FLT3-inhibitors in the treatment of FLT3-ITD acute myeloid leukemia, specificity and efficacy of NVP-HDM201 and midostaurin

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ABSTRACT

Prognosis for FLT3-ITD positive acute myeloid leukemia with high allelic ratio (>0.5) is poor, particularly in relapse, refractory to or unfit for intensive treatment, thus highlighting an unmet need for novel therapeutic approaches. The combined use of compounds targeting both the mutated FLT3 receptor and cellular p53 inhibitors might be a promising treatment option for this poor risk leukemia subset. We therefore assessed MDM2 and FLT3 inhibitors as well as cytotoxic compounds used for conventional induction treatment as single agents and in combination for their ability to induce apoptosis and cell death in leukemic cells. Acute myeloid leukemia cells represented all major morphologic and molecular subtypes with normal karyotype, including FLT3-ITD (>0.5) and FLT3 wild type, NPM1 mutant and NPM1 wild type, as well as TP53 mutant and TP53 wild type cell lines. Acute myeloid leukemia cells with mutated or deleted TP53 were resistant to MDM2- and FLT3-inhibitors. FLT3-ITD positive TP53 wild type acute myeloid leukemia cells were significantly more susceptible to FLT3-inhibitors than FLT3-ITD negative TP53 wild type cells. The presence of a NPM1 mutation reduced the susceptibility of TP53 wild type acute myeloid leukemia cells to the MDM2 inhibitor NVP-HDM201. Moreover, the combined use of MDM2- and FLT3-inhibitors was superior to single agent treatment, and the combination of midostaurin and NVP-HDM201 was as specific and effective against FLT3-ITD positive TP53 wild type cells as the combination of midostaurin with conventional induction therapy. In summary, the combined use of the MDM2 inhibitor NVP-HDM201 and the FLT3 inhibitor midostaurin was a most effective and specific treatment to target TP53 and NPM1 wild type acute myeloid leukemia cells with high allelic FLT3-ITD ratio. These data suggest that the combined use of NVP-HDM201 and midostaurin might be a promising treatment option particularly in FLT3-ITD positive acute myeloid leukemia relapsed or refractory to conventional therapy.

Introduction

Acute myeloid leukemia (AML) is a clonal hematopoietic disorder characterized by blocked differentiation and deregulated proliferation of hematopoietic precursor cells. At the cellular level, specific genetic and epigenetic alterations lead to changes in cellular signaling pathways including the common inactivation of the p53 tumor suppressor axis, and thereby contribute to blocked differentiation and accumulation of leukemic blasts in the blood and the bone marrow. The past decade has witnessed major advances in our comprehension of the biologic heterogeneity of AML.1 AML genetic variants are assigned into favorable, intermediate and poor risk categories, and a major molecular subgroup within the poor risk AML is characterized by genetic alterations of the FLT3 receptor gene. FLT3 internal tan-
dem duplications (FLT3-ITD) are the most common mutations in the FLT3 receptor gene. FLT3-mutated AML account for 25-35% of all AML, and their prognosis is poor, particularly in unfit, refractory or relapsed patients.

Targeting the mutated FLT3 receptor is a promising approach to treat this specific AML subset. Midostaurin (PKC412) is a first generation type III receptor tyrosine kinase inhibitor that has been extensively studied in vitro and in clinical trials as a treatment for AML patients with mutated FLT3. After successful phase II clinical trials, midostaurin was found to significantly prolong survival of FLT3-mutated AML patients when combined with conventional induction and consolidation therapies in a randomized phase III clinical trial leading to the first new drug approval in AML in over 40 years. Midostaurin is a multi-targeted kinase inhibitor able to block FLT3 autophosphorylation and to induce growth arrest and apoptosis in FLT3-dependent leukemia. Midostaurin is orally administered and generally well tolerated as a single agent. Quizartinib (ACC220) and gilteritinib (ASP2215) are second and third generation FLT3 inhibitors currently in evaluation for the treatment of FLT3-mutated AML.

Targeting the p53 antagonist MDM2 is a novel approach to restore the crucial p53 tumor suppressor function in AML cells. Idasanutlin (RG7388) is a second generation MDM2 inhibitor that has been studied in vitro and in vivo as a treatment for wild type TP53. NVP-CGM097 and NVP-HDM201 are second generation MDM2 inhibitors that are currently evaluated in single-agent phase I studies in patients with advanced tumors with wild type TP53 (clinicaltrials.gov identifiers 01760525 and 02143635). Like midostaurin, NVP-HDM201 is orally administered and expected to be well tolerated as single agent.

In this study, we investigated the combined treatment with MDM2- and FLT3- inhibitors, in particular NVP-HDM201 and midostaurin, on AML cells in order to identify a potentially effective treatment specifically for FLT3-ITD AML refractory to or unfit for intensive chemotherapy. The study might provide the rationale for initiating a clinical study in FLT3-ITD AML evaluating this combination.

### Methods

#### Patient samples

Mononuclear cells of AML patients diagnosed and treated at the University Hospital, Bern, Switzerland between 2005 and 2015 were included in this study. Informed consent from all patients was obtained according to the Declaration of Helsinki, and the studies were approved by decisions of the local ethics committee of Bern, Switzerland. Mutational screening for FLT3, NPM1, TP53 and conventional karyotype analysis of at least 20 metaphases were performed for each patient. Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were collected at the time of diagnosis before initiation of treatment.

#### AML cell lines

OCI-AML2 (AML-M4, FLT3wt, DNMT3A R822C, NPM1wt, TF35wt); OCI-AML3 (AML-M4, FLT3wt, DNMT3A R822C, NFM1mut, TF35wt), MOLM-13 (AML-M5, t(9;11), FLT3-ITD, TF35wt), MOLM-16 (AML-M0, FLT3wt, TP53mut), MV4-11 (AML-M5, t(4;11), FLT3-ITD, TF35wt), ML-2 (AML-M4, t(6;11), FLT3wt, TP53mut), PL-21 (AML-M3, FLT3mut, TP53hemi) and HL-60 (AML-M2, FLT3wt, TP53null) cells were supplied by the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures. AML cells were grown in RPMI 1640 (SIGMA-ALDRICH, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS, Biochrom GmbH, Germany).

#### Cytotoxicity assays

AML cells were treated with the MDM2 inhibitors NVP-HDM201, NVP-CGM097, idasanutlin (RG7388), the FLT3 inhibitors midostaurin (PKC412), quizartinib (ACC220), gilteritinib (ASP2215) or with genotoxic compounds cytarabine and idarubicin in equimolar concentrations. NVP-HDM201 and NVP-CGM097 investigational compounds were supplied by Novartis, Switzerland, whereas RG7388, PKC412, ACC220 and ASP2215 were purchased at MCE (MedChemExpress, Monmouth Junction, NJ, USA). Cytarabine and idarubicin were purchased at Sigma-Aldrich (St. Louis, MO, USA) and SelleckChem (Houston, TX, USA). Cell viability was determined using the MTT-based in vitro toxicity assay (TOX1, Sigma-Aldrich) with four repeat measurements per dosage.

### Table 1. Genetic variants in AML cell lines.

<table>
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<td>NRAS Q61L</td>
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</tr>
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wrt: wild type; ITD: internal tandem duplication; del: deletion
are depicted as XY graphs with median and interquartile range, as box plots or scatter plots with mean values. Statistical analysis was done on GraphPad Prism (version 7, GraphPad software, LaJolla, CA, USA) in grouped analysis and significance calculated by Mann-Whitney test. Combination indexes were calculated on CompuSyn software (version 1.0; ComboSyn, Inc. Paramus, NJ, USA).

Measurement of mRNA expression by qPCR
RNA was extracted from AML cells and quantified using qPCR. The RNA extraction kit was supplied by Macherey-Nagel, Düren, Germany. Reverse transcription was done with MMLV-RT (Promega, Madison, WI, USA). Real-time PCR was performed on the ABI7500 Real-Time PCR Instrument using ABI universal master mix (Applied Biosystems, Austin, TX, USA) and gene specific probes Hs00355782_m1 (CDKN1A), Hs01050896_m1 (MCL1) and Hs02758991_g1 (GAPDH) (ThermoFischer Scientific, Waltham, MA, USA). Measurements of CDKN1A and MCL1 expression were normalized with GAPDH values (ddCt relative quantitation). Assays were performed in three or more independ-

Figure 1. Variable responses of AML cell lines to FLT3 and MDM2 inhibitors. Dose response curves in AML cell lines treated with FLT3 inhibitors (A,B,C) and MDM2 inhibitors (D,E,F) as single compound treatment with midostaurin (PKC412) (A), quizartinib (AC220) (B), gilteritinib (ASP2215) (C), idasanutlin (RG7833) (D), NVP-GM097 (E) or NVP-HDM201 (F), in a variety of AML cell lines (G) and combination treatments with NVP-HDM201 and PKC412, ACC220 or ASP2215 in MOLM-13 cells (H). Combination indexes were calculated according to Chou Talalay.42
ent experiments. Statistical analysis was done on GraphPad Prism software using two-tailed t-tests (version 7, GraphPad software, LaJolla, CA, USA). Data are depicted in column bar graphs plotting mean with SD values.

**Antibodies and cytometry**

Staining for apoptosis was done using AnnexinV-CF488A (Biotium, Germany) in AnnexinV buffer and Hoechst 33342 (10 μg/ml) for 15 min. at 37°C, followed by several washes. Propidium iodide was added shortly before imaging on the Nucleocounter NC-3000 (ChemoMetec, Allerod, Denmark). For cell cycle analysis cells were incubated in lysis buffer with DAPI (10 μg/ml) for 5 min. at 37°C and analyzed on NC-3000 imager.

**Results**

**Sensitivity of AML cell lines to MDM2 and FLT3 inhibitors**

To determine the sensitivity of AML cells to MDM2 and FLT3 inhibitors, AML cell lines were treated with three MDM2- and three FLT3-inhibitors for 24 hours in dose escalation experiments before cell viability assessment. The AML cell lines covered the major morphologic and molecular subtypes including particularly FLT3-ITD and FLT3 wild type, NPM1 mutant and wild type, as well as TP53 wild type, mutant, hemizygous and null cells (Table I). The two FLT3-ITD cell lines MV4-11 and MOLM-13 had high allelic ratios of FLT3-ITD and chromosomal

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**Figure 2. Variable responses of AML blast cells to midostaurin and HDM201.** Cell viability was determined in AML patient cells treated with midostaurin (PKC412) (A) or NVP-HDM201 (B). AML cells were grouped according to major molecular subtypes (FLT3/TP53/NPM1). Cell viability measurements are depicted in dose response curves (A, B) or with 50nM single compound (C, D), as well as combination treatment of midostaurin with conventional induction therapy (E) or midostaurin with NVP-HDM201 (F). PBM are peripheral blood monocytes of normal controls. AML patient samples were analyzed in groups of at least four individual samples (Online Supplementary Table S1) using GraphPad prism software. Significance is denoted for P<0.05 (*); P<0.005 (**); P<0.0005 (***); P<0.0001 (****); P>0.05 (ns).
translocations with MLL gene rearrangements. Some cell lines contained additional mutations in driver genes such as DNMT3A (OCI-AML2, OCI-AML3) and RAS genes (OCI-AML3, PL-21). DNMT3a and RAS gene mutations may influence sensitivity to MDM2 or FLT3 inhibitors. The MDM2 inhibitors included idasanutlin (RG7388), NVP-CGM097 and NVP-HDM201. The FLT3 inhibitors included the 1st, 2nd and 3rd generation inhibitors midostaurin (PKC412), quazartinib (ACC220) and gilteritinib (ASPC2215). The FLT3-ITD positive and TP53 wild type cell lines MOLM-13 and MV4-11 were most susceptible to all three FLT3- and all three MDM2-inhibitors (Figure 1). The effects on MOLM-13 cell survival induced by the three FLT3 inhibitors were consistent with IC50 values of 200nM (Figure 1A, B, C). Quazartinib had a greater potency with 70% cell viability after treatment with 10nM compound, while midostaurin and gilteritinib had comparable potencies with 90% cell viability at 10nM compound. OCI-AML2 and PL-21 cells showed some response to midostaurin while OCI-AML3, MOLM-16 and HL-60 cells were rather resistant to midostaurin and quazartinib. With respect to gilteritinib, however, OCI-AML3, PL-21 and HL-60 cells showed some response and only MOLM-16 cells were resistant. The effects on MOLM-13 cell survival varied for the MDM2 inhibitors with IC50 values of 300nM NVP-HDM201, 1 μM RG7388 and 10 μM NVP-CGM097 (Figure 1D, E, F). MOLM-13 cells were most susceptible to MDM2 inhibitor idasanutlin with IC50 at 1 μM RG7388. MV4-11 and OCI-AML2 showed some response to idasanutlin with IC50 of 10 μM RG7388 while OCI-AML3, PL-21, HL-60 and MOLM-16 cells were rather resistant to idasanutlin. With respect to the MDM2 inhibitors NVP-CGM079 and NVP-HDM201 MOLM-13, MV4-11 and OCI-AML2 cells showed consistent susceptible responses while OCI-AML3 and PL-21 cells were less susceptible and MOLM-16 and HL60 cells were resistant. In order to define the most effective treatment combination we focused our studies on the latest and most potent MDM2 inhibitor NVP-HDM201 and tested its effects in single agent treatment and together with the three FLT3-inhibitor compounds in MOLM-13 cells. The combination of NVP-HDM201 and midostaurin had excellent synergistic effects on cell survival with a combination index of 0.4, while the combination of NVP-HDM201 with quazartinib or gilteritinib had only moderate synergistic effects with combination indexes of 0.7 and 0.8 (Figure 1F). To determine the relevance of the order of addition, midostaurin and NVP-HDM201 were tested as direct combination or sequential treatment and found to be effective independent of sequence of application. NVP-HDM201 pretreatment followed by midostaurin treatment had similar effects on cell viability as midostaurin pretreatment followed by NVP-HDM201 treatment. Moreover, both sequential treatments had comparable effects on cell viability as direct combination treatment (Online Supplementary Figure S1).

Sensitivity of AML patient cells to the MDM2 Inhibitor HDM201 and the FLT3 Inhibitor midostaurin

To determine the sensitivity of NK-AML blast cells to HDM201 and midostaurin, mononuclear cells isolated from peripheral blood or bone marrow of NK-AML patients were subjected to in vitro cytotoxicity assays. The NK-AML cells covered the major morphologic and molecular subtypes including FLT3-ITD and FLT3 wild type, NPM1 mutant and wild type, as well as TP53 mutant and wild type cells (Online Supplementary Table S1). Most of the FLT3-ITD AML cells had a high allelic ratio of FLT3-ITD (>0.5). Only few of the patient samples contained additional mutations in driver genes, one with DNMT3A, one with RAS mutations. Samples of AML blast cells were grouped according to the major molecular subtypes (FLT3/TP53/ NPM1) and comprised at least four samples per molecular genetic combination, with median 84% blast cells ranging from 25 to 95% (Online Supplementary Table S1). Both midostaurin and NVP-HDM201 used as single agent treatment induced varying levels of loss in cell viability with correlation to certain AML subsets (Figure 2). Data for MOLM-13 and MV4-11 cell lines were included.
ed as, compared to primary FLT3-ITD AML cells with 80% blast cells, FLT3-ITD AML cell lines with 100% blast cells are more susceptible to midostaurin (Figure 2A), and less susceptible to NVP-HDM201 (Figure 2B).

With respect to midostaurin, FLT3-ITD/TP53wt NK-AML cells were distinctly more susceptible than FLT3-ITD/TP53wt cells. Patient derived AML blast cells characterized by FLT3-ITD/TP53wt/NPM1wt were susceptible to midostaurin with a median loss of 30% viability after treatment with 500nM PKC412. FLT3-ITD/TP53wt/NPM1wt cells with 11q23/MLL abnormalities, MOLM-13 (t(9;11) and MV4-11 (t(4;11) lost 60% cell viability within 24 hours when treated with 500nM midostaurin. All other AML cells including FLT3-ITD/TP53wt/NPM1mut, FLT3wt and TP53mut cells were less susceptible to midostaurin with 0-10% reduced viability at 500nM midostaurin (Figure 2A).

With respect to NVP-HDM201, we observed that the same NK-AML blast cells characterized by FLT3-ITD/TP53wt/NPM1wt and sensitive to midostaurin were most susceptible to the MDM2 inhibitor NVP-HDM201, with a median loss of 45% viability within 24 hours at 100nM NVP-HDM201. MOLM-13 and MV4-11 cells were less susceptible with a loss of 20% viability at 100nM NVP-HDM201. FLT3-ITD/TP53wt/NPM1wt cells responded with a median 20% loss of viability and FLT3-ITD/TP53wt/NPM1wt with median 10% loss of viability at 100nM NVP-HDM201. All other AML cells including

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**Figure 4. Dose-dependent induction of apoptosis and cell death in FLT3-ITD AML cells.** Induction of tumor suppressor protein p53 in MV4-11 (A) and MOLM-13 cells (B) treated for 24 hours with the indicated amounts of NVP-HDM201 and midostaurin. Relative quantitation of CDKN1A mRNA (C) and MCL-1 mRNA (D) in AML cells treated for 24 hours with midostaurin (PKC412) (black bars) and NVP-HDM201 (light grey bars) alone or in combination (dark grey bars). Cytometric assays in MV4-11 AML cells treated with NVP-HDM201 and midostaurin alone and in combination to measure induction of cell death (subG1 fraction) using DAPI staining (E) and induction of apoptosis using AnnexinV/PI staining (F).
FLT3-ITD/TP53wt/NPM1wt and TP53mut AML cells were minimally susceptible to NVP-HDM201 with 1-5% reduced viability when treated with 1 μM compound (Figure 2B). In single agent treatments with 50nM compound, FLT3-ITD/TP53wt/NPM1wt cells were significantly more susceptible to midostaurin and NVP-HDM201 than FLT3-ITD/TP53mt/NPM1mut and FLT3wt cells (Figure 2C,D). FLT3-ITD/TP53wt/NPM1wt cells lost more than 20% cell viability within 24 hours at 50nM PKC412 while all other AML cells and normal peripheral blood monocytes were unaffected by this low dose treatment (Figure 2C). Similarly, FLT3-ITD/TP53wt/NPM1wt cells lost 30% cell viability within 24 hours at 50nM NVP-HDM201 while all other AML cells were significantly less affected by this low dose treatment and TP53mut AML cells as well as normal peripheral blood monocytes were unaffected (Figure 2D).

These data propose that the ideal target population for the treatment with midostaurin and NVP-HDM201 are FLT3-ITD NK-AML cells with a high allelic ratio of FLT3-ITD that are wild type for TP53 and NPM1.

Specificity and efficacy of combined HDM201 and midostaurin against FLT3-ITD/TP53wt/NPM1wt AML cells

The response of AML cells to 50nM midostaurin in combination with conventional induction therapy (CI, 20nM cytarabine and 20nM idarubicin) or in combination with 50nM NVP-HDM201 was determined by in vitro cytotoxicity assays. Similar to the single agent treatments reported above, FLT3-ITD/TP53wt NK-AML cells were most susceptible to the combined treatment whereas FLT3-ITD/TP53mut cells turned out to be resistant and FLT3-ITD/TP53wt cells showed intermediate responses (Figure 2E,F).

The combination of midostaurin with conventional induction treatment had significant effects on TP53wt AML cells with 30-40% median loss of cell viability in FLT3-ITD cells and 15-25% reduction in FLT3wt cells exposed to 20nM CI and 50nM midostaurin for 24 hours (Figure 2E). TP53mut AML cells and normal peripheral blood monocytes were affected with 5-10% median loss in cell viability. The effects of conventional induction treatment on cell viability were enhanced by the addition of midostaurin in FLT3-ITD/TP53wt cells independent of their NPM1 status (Figure 2E).

The combination of midostaurin with NVP-HDM201 was as effective as the combination of midostaurin with standard induction therapy. As in the single agent treatments, FLT3-ITD/TP53wt/NPM1wt cells were most susceptible to this combination with 40% median loss of cell viability in 24 hours to 50nM NVP-HDM201 and 50nM midostaurin (Figure 2F). FLT3-ITD/TP53wt/NPM1mut and FLT3wt/TP53wt/NPM1wt AML cells were less susceptible with a median reduction of 20% cell viability in this combination treatment. FLT3wt/TP53wt/NPM1mut and TP53mut AML cells as well as normal peripheral blood monocytes were least affected with 8% median losses in cell viability. The combination of NVP-HDM201 and midostaurin had synergistic effects on cell survival of FLT3-ITD positive AML cells with a combination index of 0.63 in the relapsed AML patient #13 (Figure 3A), and moderate synergistic effects in FLT3-ITD positive primary AML cells (Figure 3B,C). FLT3-ITD/TP53wt/NPM1wt primary AML cells (patient #16) were most susceptible to this combination treatment (Figure 3B) with reduced responses in FLT3-ITD/TP53wt/NPM1wt (patient#20) primary AML (Figure 3C).

To confirm p53 activation in the presence of MDM2 inhibitors we determined the expression levels of the tumor suppressor protein p53 and of the p53 target genes CDKN1A and MCL1 in AML cells treated for 24 hours with single compounds and with combined treatment. Protein p53 was stabilized and p53 levels were increased in AML cells treated with 200nM NVP-HDM201, with three- to eightfold induction in MV4-11, MOLM-13 and OCI-AML3 cells, while OCI-AML2 cells had a high p53 level with a maximal 20% increase (Figure 4 A, B, C, D). CDKN1A gene expression was significantly induced in FLT3-ITD/TP53wt/NPM1wt AML cells (MOLM-13, MV4-11, patient#15) and in FLT3wt/TP53wt/NPM1wt cells (OCI-AML2) treated with 50nM NVP-HDM201 (Figure 4E), and in FLT3wt/TP53wt/NPM1mut cells (OCI-AML3) treated with 500nM NVP-HDM201, but not in FLT3wt/TP53mut cells (MOLM-16). To reach the same level of p53 target gene expression induced by 50nM NVP-HDM201 in FLT3-ITD/NPM1wt (MOLM-13, MV4-11, pat#13) and FLT3wt/NPM1wt (OCI-AML2) cells, ten times more MDM2 inhibitor was required in FLT3wt/NPM1mut (OCI-AML3) cells. Yoshimoto et al. 2009 showed that FLT3-ITD up-regulates the apoptosis inhibitor MCL-1 to promote survival of stem cells in acute myeloid leukemia. They analyzed the function of MCL-1 in FLT3-ITD AML and showed that the enforced expression of MCL-1 prevented MV4-11 cells from apoptosis in the presence of 100nM midostaurin. Inhibition of MCL-1 by shRNA resulted in apoptosis of MV4-11 cells. To elucidate the mechanism of apoptosis induction by NVP-HDM201 and midostaurin we analyzed MCL-1 expression in a variety of AML cells (Figure 4F). MCL-1 gene expression was repressed in the presence of 50nM NVP-HDM201 or 50nM midostaurin in FLT3-ITD/TP53wt/NPM1wt AML cells (MOLM-13, MV4-11, patient#15), with enhanced effects in the combination treatments. MCL-1 gene expression was repressed in the presence of 50nM NVP-HDM201 in FLT3wt/TP53wt/NPM1wt cells (OCI-AML2) and by 500nM NVP-HDM201 in FLT3wt/TP53wt/NPM1mut cells (OCI-AML3), but not by midostaurin. There was no repression of MCL-1 gene expression in FLT3wt/TP53mut cells (MOLM-16). In the susceptible FLT3-ITD cell lines MV4-11 and MOLM-13 as well as in the relapsed FLT3-ITD AML sample (patient#15) both compounds led to a significant reduction in MCL-1 gene expression with enhanced reduction in the combination treatments (Figure 4F). The effect of NVP-HDM201 and midostaurin treatment on MCL-1 gene repression appeared to be strongly synergistic with a combination index of 0.25. To further assess pro-apoptotic effects in AML cells treated with midostaurin and with the MDM2 inhibitor NVP-HDM201, cells were stained with AnnexinV and DAPI and analyzed on a cell imager. Apoptosis and cell death were induced in FLT3-ITD/TP53wt/NPM1wt cells in a dose dependent manner by both inhibitors in single compound and combination treatments. There was a significant increase in dead cells with subG1 DNA content, and a concomitant loss of cells in defined cell cycle stages, most prominently a reduction of cells with G0/G1 phase DNA content, but also of cells with S-phase and G2 phase
content in MV4-11 cells treated with 20nM PKC412 and 50nM NVP-HDM201 (Figure 4G). Moreover, there was a significant increase in the number of AnnexinV positive apoptotic cells and a concomitant reduction in AnnexinV negative non-apoptotic cells in MV4-11 cells treated with 20nM PKC412 and 50nM NVP-HDM201 (Figure 4H). The pro-apoptotic and lethal effects of the single compound treatments were enhanced in the combined treatments with a combination index of 0.44 indicating synergistic pro-apoptotic and lethal effects with NVP-HDM201 and midostaurin. A similar induction of apoptosis and cell death was also detected in MOLM-13 cells treated with 20nM PKC412 and 50nM NVP-HDM201 (Online Supplementary Figure S2). While there was no pro-apoptotic effect in FLT3wt/TP53wt/NPM1mut (OCI-AML3) and FLT3-ITD/TP53mut (PL-21) cells at 100nM compounds, a low-level induction of apoptosis and cell death was detected in OCI-AML3 and PL-21 cells after 24 hours of treatment with 1 μM compounds (data not shown).

In summary, our data indicate that NVP-HDM201 and midostaurin can induce apoptosis and cell death effectively and specifically in FLT3-ITD/TP53wt/NPM1wt AML cells. FLT3-ITD is a constitutively active growth factor receptor signaling via PI3K-AKT, via RAS-MEK-ERK and via STAT5 leading to cell growth and proliferation via p53 inhibition and MCL1 induction (Figure 5). We have shown that MDM2 inhibition by NVP-HDM201 can reactivate p53 function leading to induction of CDKN1A and inhibition of MCL1 gene expression. Inhibition of FLT3-ITD by midostaurin, however, did not restore p53 function, but led to reduced MCL1 gene expression via RAS-MEK-ERK and/or STAT5 signaling (Figure 5). These data suggest that the combined use of NVP-HDM201 and midostaurin might be a promising treatment option particularly in FLT3-ITD AML relapsed or refractory to conventional therapy.

Discussion

Acute myeloid leukemia (AML) characterized by normal karyotype (NK) and presence of the mutated FLT3 growth factor receptor gene variant FLT3-ITD comprises 27-34% of newly diagnosed AML. The subset of NK-AML patients with high allelic ratio of FLT3-ITD (>0.5) and NPM1 wild type is associated with adverse risk and low-

![Figure 5. Schematic representation of the FLT3-ITD signaling pathways and downstream effects. FLT3-ITD is a constitutively active growth factor receptor signaling via PI3K-AKT, via RAS-MEK-ERK and via STAT5 leading to cell growth and proliferation via p53 inhibition and MCL1 induction. p53 function can be reactivated by NVP-HDM201 treatment leading to induction of CDKN1A and inhibition of MCL1 gene expression. MCL1 gene expression can be inhibited by NVP-HDM201 via p53 induction and by midostaurin (PKC412) via RAS-MEK-ERK and/or STAT5 signaling.](image-url)
est survival rates. Survival rates are higher in NK-AML patients with high allelic ratio of FLT3-ITD and NPM1mut or low allelic ratio of FLT3-ITD and NPM1 wild type or FLT3wt and NPM1mut which have all been classified as intermediate risk.39 The leukemic cells of all these AML subsets have substantially elevated levels of cellular p53 antagonists and reduced p53 activity17 which identifies them as targets for treatments aiming to restore p53 function including conventional chemotherapy with genotoxic compounds and non-genotoxic treatments with p53 reactivating compounds.

MDM2 is an established cellular p53 antagonist frequently overexpressed in AML cells. A variety of MDM2 inhibitors have been developed and tested in AML cell lines. Compounds currently in clinical trials for the treatment of AML include idasanutlin (RG7388), NVP-CGM097 and NVP-HDM201.12,13,14 We have studied the MDM2 inhibitor idasanutlin in combination with the MEK inhibitor cobimetinib in AML cell lines and patient samples and found this combination to be effective only in AML cells expressing high levels of FLT3 and MDM2 protein.20 The activity of NVP-CGM097 was investigated in AML cell lines and primary AML cells expressing wild type and mutant p53, alone and in combination with the FLT3 inhibitor PKC412 (midostaurin) or the MEK inhibitor AZD 6244.17 Synergy was observed when NVP-CGM097 was combined with FLT3 inhibition against oncogenic FLT3 expressing cells, as well as when combined with MEK inhibition in cells with activated MAPK signalling. In addition to reactivating p53 in AML cells by specific MDM2 inhibition the FLT3 receptor can be directly targeted by (more or less) specific tyrosine kinase inhibitors.25

In the present study, we tested a variety of FLT3 and MDM2 inhibitors. The effects on cell survival of FLT3-ITD AML cells were consistent for the FLT3 inhibitors midostaurin (PKC412), quizzartinib (ACC220) and gilteritinib (ASP2215), but varied for the MDM2 inhibitors idasanutlin (RG7388), NVP-CGM097 and NVP-HDM201. The most potent MDM2 inhibitor NVP-HDM201 exhibited superior combinatory effects on cell viability of FLT3-ITD AML cells together with midostaurin, and moderate combinatory effects together with quizzartinib and gilteritinib. The different combinatory potentials may be related to the target specificity of the three FLT3 inhibitors. While quizzartinib (ACC220) inhibits FLT3 and PDGFR kinases,22 gilteritinib (ASP2215) inhibits FLT3, LTK, ALK, and AXL kinases, and midostaurin (PKC412) inhibits FLT3, KIT, PCK, PPK, VEGFR-2, PDGFR, and SYK kinases.23 PDGFR and VEGFR-2 are expressed in the bone marrow of AML patients24,25 and, like FLT3, signal via PI3K/AKT and MDM2 to inhibit p53.26-27 The stem cell growth factor receptor KIT is expressed in the bone marrow and, like FLT3-ITD, signals via PI3K/AKT and MDM2 to inhibit p53, and via JAK2 and STAT5.28

MOLM-13 and MV4-11 AML cells were most susceptible to the FLT3 inhibitor midostaurin. Both cell lines have a high allelic ratio of FLT3-ITD and harbor MLL rearrangements, created by t(9;11) and t(4;11), encoding MLL-AF9 and MLL-ENL, respectively. FLT3 and MLL collaborate in AML29 and leukemic cells with mutations in FLT3 and MLL are known to be susceptible to midostaurin.30,31 In the absence of MLL mutations, FLT3-ITD AML cells were less susceptible to midostaurin, but more susceptible to NVP-HDM201 indicating that the presence of MLL fusion pro-
with newly diagnosed acute myeloid leukemia (AML) who are FLT3 mutation-positive (FLT3+), in combination with standard cytarabine and daunorubicin induction and cytarabine consolidation. This combination therapy is, however, not suitable for NK AML patients with FLT3-ITD in relapse or refractory to conventional induction treatment or unfit for intensive treatment. For the subset of AML patients with a high ratio of FLT3-ITD and adverse prognosis, the combined use of non-genotoxic targeted compounds, such as the combination of midostaurin and NVP-HDM201, may represent a promising treatment option. Synergistic effects on cell viability with midostaurin and NVP-HDM201 were observed independent of sequence of application, indicating that the order of target inhibition for FLT3 and MDM2 was not important. Sequential application of NVP-HDM201 and midostaurin had the same effects on cell viability as direct combination treatment. Pretreatment with one inhibitor did not enhance the susceptibility of AML cells to the second inhibitor. This leaves several options for treatment regimens in a prospective clinical trial. The best therapy regimen in combination treatments with midostaurin and NVP-HDM201 for AML patients will have to be defined empirically.

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References


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