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

Katja Seipel,<sup>1,2</sup> Miguel A.T. Marques,<sup>1</sup> Corinne Sidler,<sup>1</sup> Beatrice U. Mueller<sup>2</sup> and Thomas Pabst<sup>2</sup>

<sup>1</sup>Department for Biomedical Research, University of Bern and <sup>2</sup>Department of Medical Oncology, Inselspital, Bern University Hospital, Switzerland

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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.

## Correspondence:

thomas.pabst@insel.ch

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## 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.<sup>1</sup> 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*.<sup>2,3</sup> 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.<sup>4</sup> Midostaurin is a multi-targeted kinase inhibitor able to block *FLT3* autophosphorylation and to induce growth arrest and apoptosis in *FLT3*-dependent leukemia.<sup>5</sup> 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.<sup>6-8</sup>

Targeting the p53 antagonist MDM2 is a novel approach to restore the crucial p53 tumor suppressor function in AML cells.<sup>9</sup> Idasanutlin (RG7833) is a second generation MDM2 inhibitor that has been studied *in vitro* and *in vivo* as a treatment for AML patients with wild type TP53.<sup>10</sup> NVP-CGM097<sup>11</sup> and NVP-HDM201<sup>12</sup> 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, *FLT3*wt, DNMT3A R882C, *NPM1*wt, *TP53*wt); OCI-AML3 (AML-M4, *FLT3*wt, DNMT3A R882C, *NPM1*mut, *TP53*wt), MOLM-13 (AML-M5, t(9;11), *FLT3*-ITD, *TP53*wt), MOLM-16 (AML-M0, *FLT3*wt, *TP53*mut), MV4-11 (AML-M5, t(4;11), *FLT3*-ITD, *TP53*wt), ML-2 (AML-M4, t(6;11), *FLT3*wt, *TP53*mut), PL-21 (AML-M3, *FLT3*wt, *TP53*hemi) and HL-60 (AML-M2, *FLT3*wt, *TP53*null) 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 (RG7833), 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 RG7833, 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* toxicology assay (TOX1, Sigma-Aldrich) with four repeat measurements per dosage. Data

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

| ID       | <i>FLT3</i> | <i>TP53</i> | <i>NPM1</i> | mutated genes              |
|----------|-------------|-------------|-------------|----------------------------|
| HL-60    | wt          | del         | wt          | NRAS Q61L<br>CDKN2A R80X   |
| MOLM-13  | ITD         | wt          | wt          | MLL-AF9 (t9;11)            |
| MOLM-16  | wt          | V173M C238S | wt          | MLL V1368L<br>MTOR T571K   |
| MV4-11   | ITD         | wt          | wt          | MLL-ENL (t4;11)            |
| OCI-AML2 | wt<br>A680V | wt          | wt          | DNMT3A R635W<br>MLL K1751* |
| OCI-AML3 | wt          | wt          | L287fs      | DNMT3A R882C<br>NRAS Q61L  |
| PL-21    | wt<br>P336L | wt<br>P36fs | wt          | KRAS A146V                 |

wt: 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-

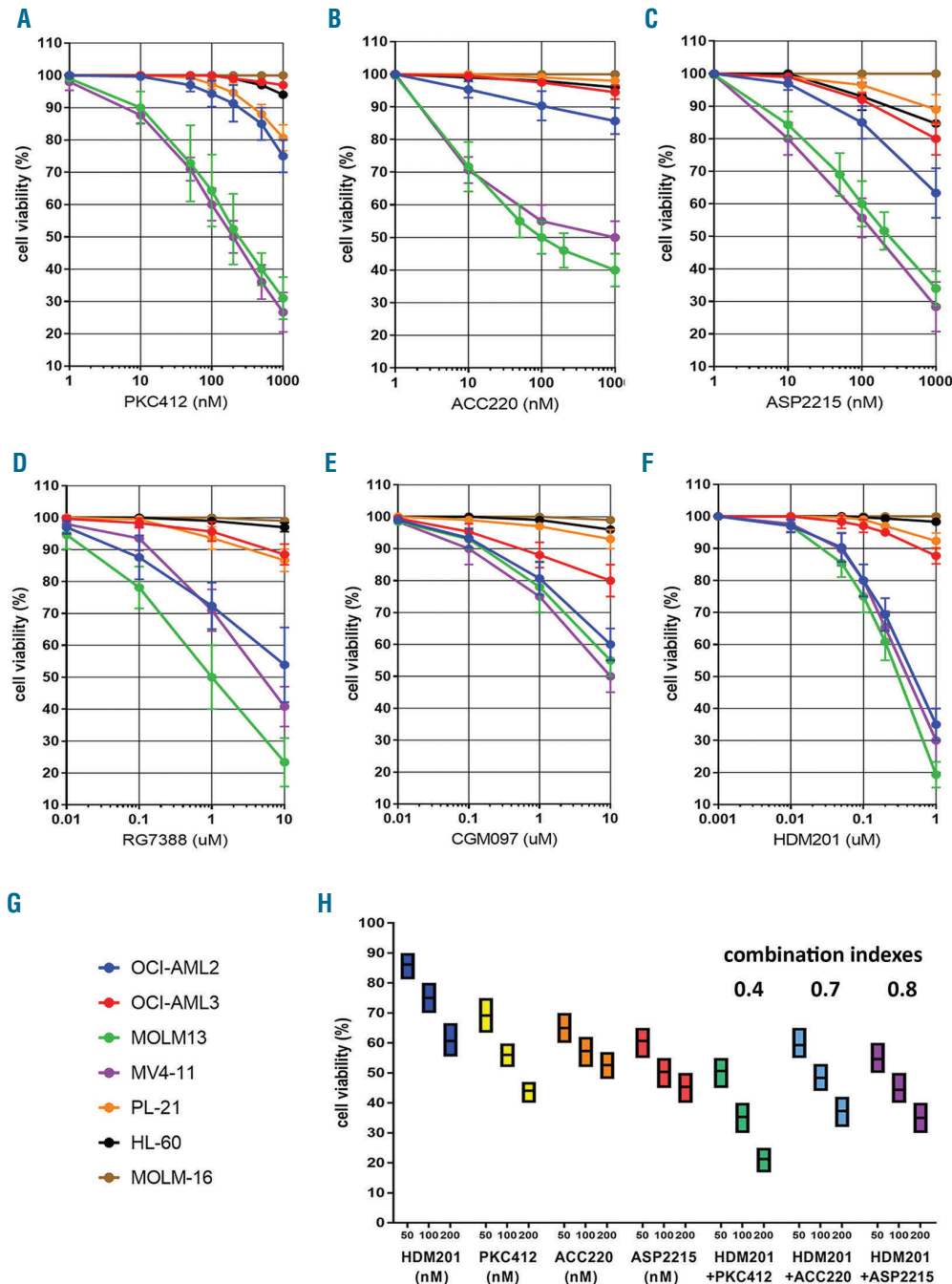


Figure 1. Variable responses of AML cell lines to FLT3 and MDM2 inhibitors. Dose response curves in AML cell lines treated with FLT3 inhibitors (A,B,C) and MDM2 inhibitors (D,E,F) as single compound treatment with midostaurin (PKC412) (A), quizartinib (ACC220) (B), gilteritinib (ASP2215) (C), idasanutlin (RG7383) (D), NVP-CGM097 (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.<sup>42</sup>

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.

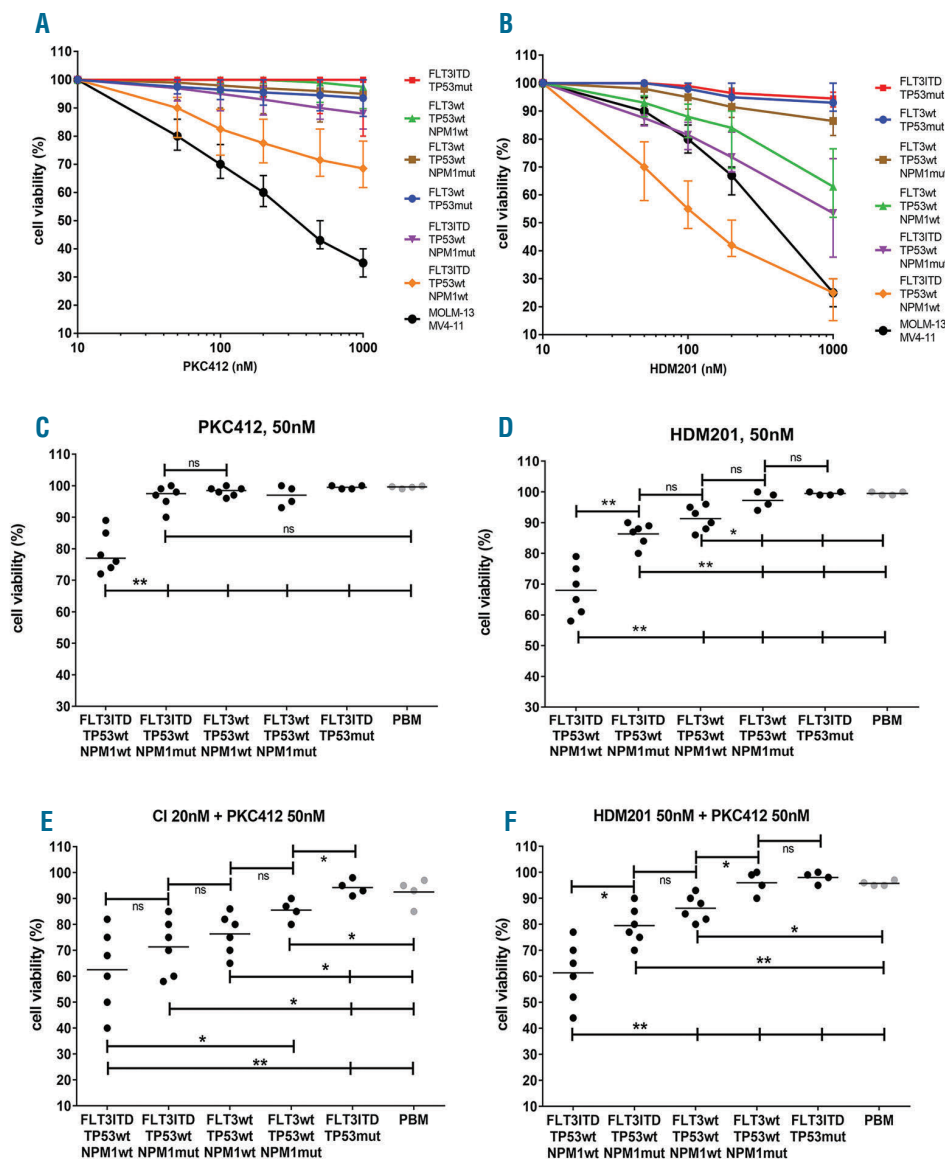
### Antibodies and cytometry

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, Allerød, 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 1). The two *FLT3*-ITD cell lines MV4-11 and MOLM-13 had high allelic ratios of *FLT3*-ITD and chromosomal



**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).

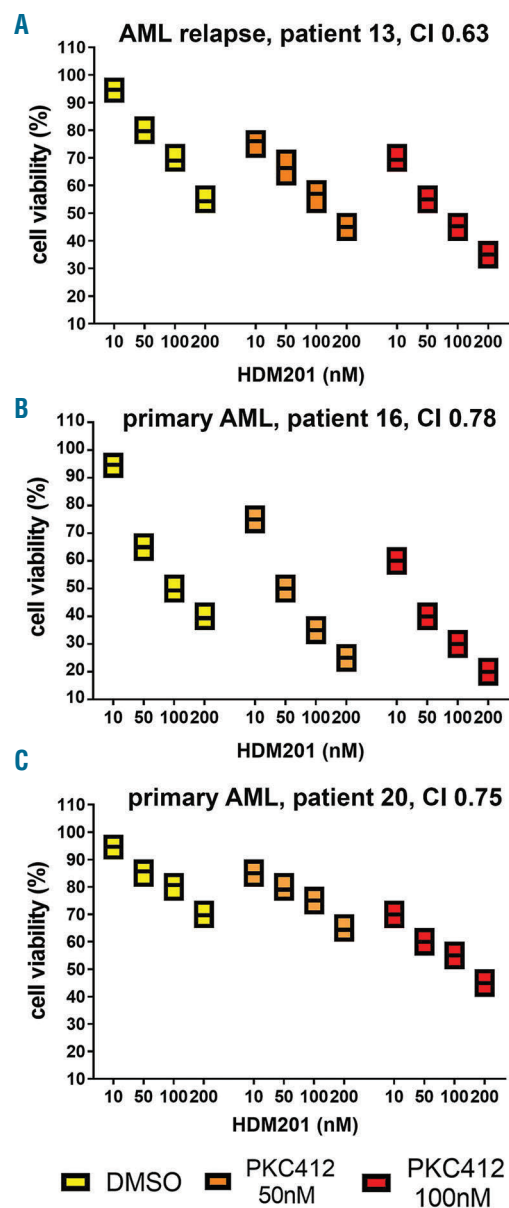


translocations with MLL gene rearrangements. Some cell lines contained additional mutations in driver genes such as *DNMT3A* (OCI-AML2, OCI-AML3) and *RAS* genes (OCI-AML3, PL-21). DNMT3a and *RAS* gene mutations may influence sensitivity to MDM2 or *FLT3* inhibitors. The MDM2 inhibitors included idasanutlin (RG7388), NVP-CGM097 and NVP-HDM201. The *FLT3* inhibitors included the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> generation inhibitors midostaurin (PKC412), quizartinib (ACC220) and gilteritinib (ASP2215). 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 IC<sub>50</sub> values of 200nM (Figure 1A,B,C). Quizartinib 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 quizartinib. 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 IC<sub>50</sub> 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 IC<sub>50</sub> at 1 μM RG7388. MV4-11 and OCI-AML2 cells showed some response to idasanutlin with IC<sub>50</sub> 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-CGM097 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 quizartinib or gilteritinib had only moderate synergistic effects with combination indexes of 0.7 and 0.8 (Figure 1H). 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 includ-



**Figure 3. Synergistic responses in *FLT3*-ITD AML blast cells to midostaurin and HDM201.** Cell viability was determined in individual AML patient cells treated with increasing dosage of NVP-HDM201 as single compound and in combination treatment with midostaurin (PKC412) in *FLT3*-ITD/*TP53*wt/*NPM1*wt relapsed AML (patient 13) (A), *FLT3*-ITD/*TP53*wt/*NPM1*wt primary AML (patient 16) (B) and in *FLT3*-ITD/*TP53*wt/*NPM1*mut primary AML (patient 20) (C). Combination indexes were calculated according to Chou Talalay.<sup>42</sup>

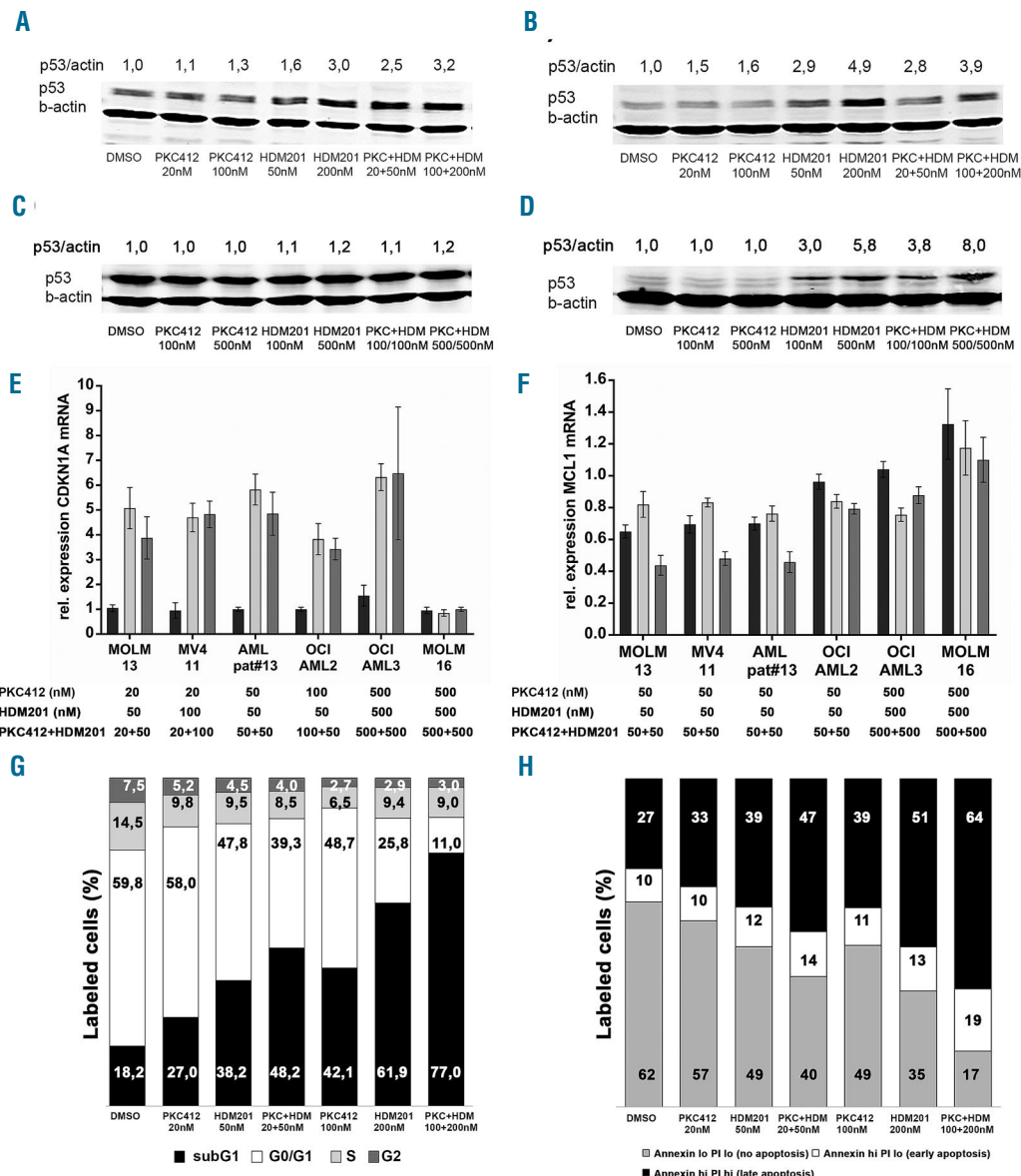
ed as, compared to primary *FLT3*-ITD AML cells with 80% blast cells, *FLT3*-ITD AML cell lines with 100% blast cells are more susceptible to midostaurin (Figure 2A), and less susceptible to NVP-HDM201 (Figure 2B).

With respect to midostaurin, *FLT3*-ITD/*TP53*wt NK-AML cells were distinctly more susceptible than *FLT3*-ITD/*TP53*wt cells. Patient derived AML blast cells characterized by *FLT3*-ITD/*TP53*wt/*NPM1*wt were susceptible to midostaurin with a median loss of 30% viability after treatment with 500nM PKC412.

*FLT3*-ITD/*TP53*wt/*NPM1*wt 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/*TP53*wt/*NPM1*mut, *FLT3*wt and *TP53*mut 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/*TP53*wt/*NPM1*wt 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/*TP53*wt/*NPM1*wt cells responded with a median 20% loss of viability and *FLT3*-ITD/*TP53*wt/*NPM1*wt with median 10% loss of viability at 100nM NVP-HDM201. All other AML cells including



**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 *MCL1* 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/*TP53*wt/*NPM1*wt and *TP53*mut cells were minimally susceptible to NVP-HDM201 with 1-5% reduced viability when treated with 1  $\mu$ M compound (Figure 2B).

In single agent treatments with 50nM compound, *FLT3*-ITD/*TP53*wt/*NPM1*wt cells were significantly more susceptible to midostaurin and NVP-HDM201 than *FLT3*-ITD/*TP53*wt/*NPM1*mut and *FLT3*wt cells (Figure 2C,D). *FLT3*-ITD/*TP53*wt/*NPM1*wt 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/*TP53*wt/*NPM1*wt 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 *TP53*mut 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/*TP53*wt/*NPM1*wt 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/*TP53*wt NK-AML cells were most susceptible to the combined treatment whereas *FLT3*-ITD/*TP53*mut cells turned out to be resistant and *FLT3*-ITD/*TP53*wt cells showed intermediate responses (Figure 2E,F).

The combination of midostaurin with conventional induction treatment had significant effects on *TP53*wt AML cells with 30-40% median loss of cell viability in *FLT3*-ITD cells and 15-25% reduction in *FLT3*wt cells exposed to 20nM CI and 50nM midostaurin for 24 hours (Figure 2E). *TP53*mut AML cells and normal peripheral blood monocytes were affected with 5-10% median losses in cell viability. The effects of conventional induction treatment on cell viability were enhanced by the addition of midostaurin in *FLT3*-ITD/*TP53*wt 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/*TP53*wt/*NPM1*wt 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/*TP53*wt/*NPM1*mut and *FLT3*wt/*TP53*wt/*NPM1*wt AML cells were less susceptible with a median reduction of 20% cell viability in this combination treatment. *FLT3*wt/*TP53*wt/*NPM1*mut and *TP53*mut AML cells as well as normal peripheral blood monocytes were least affected with 3% 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/*TP53*wt/*NPM1*wt pri-

mary AML cells (patient #16) were most susceptible to this combination treatment (Figure 3B) with reduced responses in *FLT3*-ITD/*TP53*wt/*NPM1*wt (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/*TP53*wt/*NPM1*wt AML cells (MOLM-13, MV4-11, patient#13) and in *FLT3*wt/*TP53*wt/*NPM1*wt cells (OCI-AML2) treated with 50nM NVP-HDM201 (Figure 4E), and in *FLT3*wt/*TP53*wt/*NPM1*mut cells (OCI-AML3) treated with 500nM NVP-HDM201, but not in *FLT3*wt/*TP53*mut cells (MOLM-16). To reach the same level of p53 target gene expression induced by 50nM NVP-HDM201 in *FLT3*-ITD/*NPM1*wt (MOLM-13, MV4-11, pat#13) and *FLT3*wt/*NPM1*wt (OCI-AML2) cells, ten times more MDM2 inhibitor was required in *FLT3*wt/*NPM1*mut (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/*TP53*wt/*NPM1*wt AML cells (MOLM-13, MV4-11, patient#13), with enhanced effects in the combination treatments. MCL-1 gene expression was repressed in the presence of 50nM NVP-HDM201 in *FLT3*wt/*TP53*wt/*NPM1*wt cells (OCI-AML2) and by 500nM NVP-HDM201 in *FLT3*wt/*TP53*wt/*NPM1*mut cells (OCI-AML3), but not by midostaurin. There was no repression of MCL-1 gene expression in *FLT3*wt/*TP53*mut 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#13) 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/*TP53*wt/*NPM1*wt 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



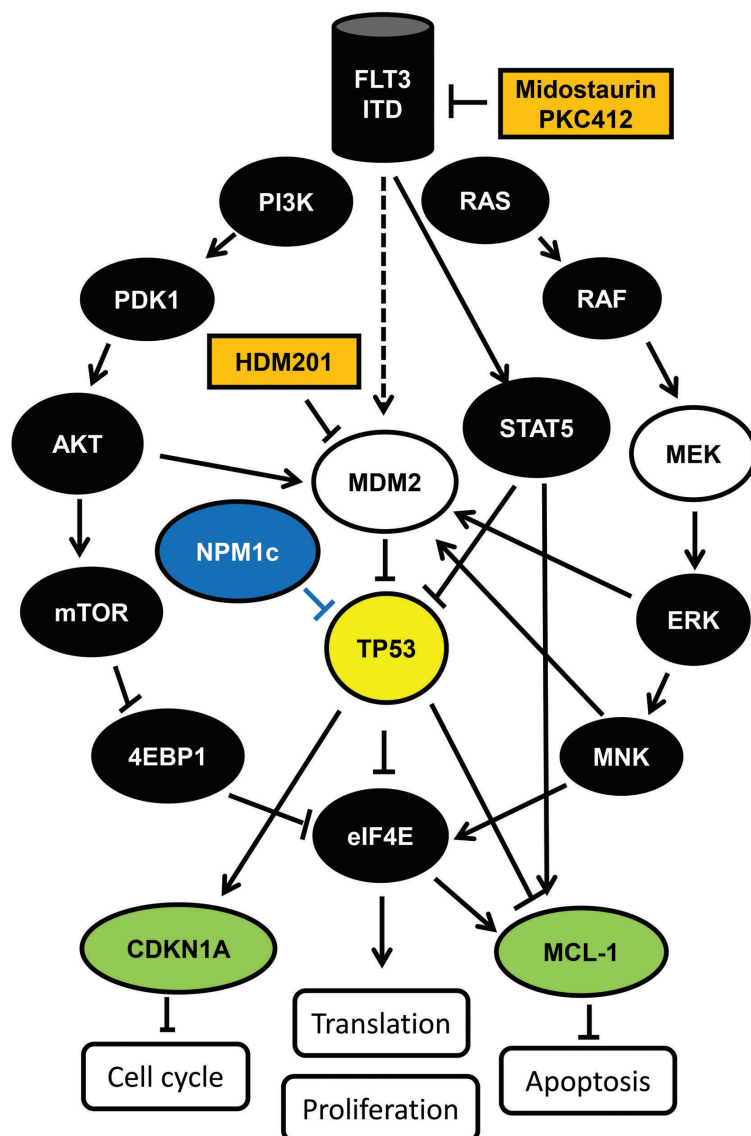
content in *MV4-11* cells treated with 20nM PKC412 and 50nM NVP-HDM201 (Figure 4G). Moreover, there was a significant increase in the number of AnnexinV positive apoptotic cells and a concomitant reduction in AnnexinV negative non-apoptotic cells in *MV4-11* cells treated with 20nM PKC412 and 50nM NVP-HDM201 (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 *FLT3*wt/*TP53*wt/*NPM1*mut (OCI-AML3) and *FLT3*-ITD/*TP53*mut (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  $\mu$ M 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/*TP53*wt/*NPM1*wt AML cells. *FLT3*-ITD is a constitutively active growth factor

receptor signaling via PI3K-AKT,<sup>13</sup> via RAS-MEK-ERK<sup>13</sup> and via STAT5<sup>14,15</sup> 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.



est survival rates. Survival rates are higher in NK-AML patients with high allelic ratio of *FLT3*-ITD and *NPM1*mut or low allelic ratio of *FLT3*-ITD and *NPM1* wild type or *FLT3*wt and *NPM1*mut which have all been classified as intermediate risk.<sup>16</sup> The leukemic cells of all these AML subsets have substantially elevated levels of cellular p53 antagonists and reduced p53 activity<sup>17</sup> 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.<sup>12,18,19</sup> 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.<sup>20</sup> 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.<sup>19</sup> 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.<sup>5,21</sup>

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), quizartinib (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 quizartinib and gilteritinib. The different combinatory potentials may be related to the target specificity of the three *FLT3* inhibitors. While quizartinib (ACC220) inhibits *FLT3* and PDGFR kinases,<sup>22</sup> gilteritinib (ASP2215) inhibits *FLT3*, LTK, ALK, and AXL kinases,<sup>8</sup> and midostaurin (PKC412) inhibits *FLT3*, KIT, PKC, PPK, VEGFR-2, PDGFR, and SYK kinases.<sup>23</sup> PDGFR and VEGFR-2 are expressed in the bone marrow of AML patients<sup>24,25</sup> and, like *FLT3*, signal via PI3K/AKT and MDM2 to inhibit p53.<sup>26,27</sup> 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.<sup>28</sup>

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 cooperate in AML<sup>29</sup> and leukemic cells with mutations in *FLT3* and MLL are known to be susceptible to midostaurin.<sup>30,31</sup> 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-

teins may change the susceptibility of AML cells to *FLT3* and MDM2 inhibitors.

NK-AML cells with *FLT3*-ITD/*TP53*wt/*NPM1*wt were particularly susceptible to the combination of midostaurin with conventional induction therapy or with NVP-HDM201. In contrast, NK-AML cells with mutated *TP53* were rather resistant to midostaurin and NVP-HDM201, and they have previously been shown to be resistant to chemotherapy with genotoxic compounds<sup>32</sup> and to other MDM2 inhibitors.<sup>33</sup> As both the conventional induction therapy and MDM2 inhibition induce cell cycle exit and apoptosis via p53 activation, these treatments can only be effective in a *TP53* wild type context.<sup>9</sup> The presence of one mutated *TP53* allele may be sufficient to suppress the function of the remaining p53 wild type protein. Hence, restoring wild-type p53 activity may be a promising option for treatment of AML with mutated *TP53*.<sup>34</sup> HL-60 cells with deleted *TP53* were resistant to midostaurin and HDM201, indicating that these two compounds specifically target AML cells with functional p53 protein. This is in apparent contrast to the *FLT3*-inhibitor sorafenib and the MDM2 inhibitor nutlin-3 which promoted synergistic cytotoxicity irrespectively of *FLT3* and p53 status via induction of the pro-apoptotic Bcl-2 family members Bax and Bak in p53 wild type and p53 deleted cells.<sup>35</sup>

It seems remarkable that *FLT3*wt/*TP53*wt/*NPM1*mut AML cells turned out to be as refractory to midostaurin and NVP-HDM201 as *TP53*mut cells. This suggests that the presence of mutated *NPM1* protein is sufficient to prevent induction of p53 target genes by midostaurin or NVP-HDM201 in these cells. The mutated *NPM1* protein (*NPM1c*) reduces the susceptibility of *FLT3*-ITD AML cells to both midostaurin and NVP-HDM201, indicating that the same molecular mechanisms may be involved in *FLT3*wt and *FLT3*-ITD cells. *NPM1c* is exported to the cytoplasm<sup>36</sup> where it inhibits the tumor suppressor protein p53 by cytoplasmic retention. This leads to reduction of p53 protein in the nucleus and decreases its stability and transcriptional activation function.<sup>37</sup> It appears that this loss of p53 activity by *NPM1c* cannot be compensated by inhibition of MDM2 or *FLT3*, at least not with NVP-HDM201 or midostaurin. In contrast, nutlin-3 can induce apoptosis and cell death in *FLT3*wt/*TP53*wt/*NPM1*mut cells,<sup>17</sup> indicating a fundamental difference in the activities of the two MDM2 inhibitors.

The molecular mechanisms involved in p53 activation appear to be different in the conventional induction treatment. Here, *FLT3*wt/*TP53*wt/*NPM1*mut AML cells were more susceptible to conventional induction treatment than *TP53*mut cells, indicating that the presence of *NPM1c* is not sufficient to prevent p53 activation by genotoxic substances. This may be reflected by the usually favorable outcome of *FLT3*wt/*TP53*wt/*NPM1*mut AML patients after standard induction treatment.<sup>38,39</sup> Midostaurin can enhance effects of conventional induction therapy in *FLT3*-ITD cells in vitro and in vivo. As evaluated in a randomized, double-blind, phase III international study ([clinicaltrials.gov/identifer/00651261](http://clinicaltrials.gov/identifer/00651261)) in high-risk patients with newly diagnosed, *FLT3*-mutant AML (CALGB10603/RATIFY trial), the multikinase inhibitor midostaurin plus standard chemotherapy improved survival compared with placebo plus chemotherapy.<sup>40,41</sup> On April 28, 2017, the U.S. Food and Drug Administration approved midostaurin (RYDAFT, Novartis Pharmaceuticals Corp.) for the treatment of adult patients

with newly diagnosed acute myeloid leukemia (AML) who are FLT3 mutation-positive (FLT3<sup>+</sup>), 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

- Sweet K, Lancet J. State of the art update and next questions: acute myeloid leukemia. *Clin Lymphoma Myeloma Leuk*. 2017;17(11):703-709.
- Fischer T, Stone RM, Deangelo DJ, et al. Phase IIB trial of oral Midostaurin (PKC412), the FMS-like tyrosine kinase 3 receptor (FLT3) and multi-targeted kinase inhibitor, in patients with acute myeloid leukemia and high-risk myelodysplastic syndrome with either wild-type or mutated FLT3. *J Clin Oncol*. 2010; 28(28):4339-4345.
- Gallooly MM, Lazarus HM, Cooper BW. Midostaurin: a novel therapeutic agent for patients with FLT3-mutated acute myeloid leukemia and systemic mastocytosis. *Ther Adv Hematol*. 2017;8(9):245-261.
- Levis M. Midostaurin approved for FLT3-mutated AML. *Blood*. 2017; 129(26):3403-3406.
- Weisberg E, Boulton C, Kelly LM, et al. Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. *Cancer Cell*. 2002;1(5):433-443.
- Levis M. Quizartinib in acute myeloid leukemia. *Clin Adv Hematol Oncol*. 2013; 11(9):586-588.
- Hills RK, Gammon G, Trone D, Burnett AK. Quizartinib significantly improves overall survival in FLT3-ITD positive AML patients relapsed after stem cell transplantation or after failure of salvage chemotherapy: a comparison with Historical AML Database (UK NCRI data). *Blood*. 2015; 126(23):2557-2557.
- Ueno Y, Mori M, Kamiyama Y, Kaneko N, Isshiki E, Takeuchi M. Gilteritinib (ASP2215), a novel FLT3/AXL inhibitor: preclinical evaluation in combination with azacitidine in acute myeloid leukemia. *Blood*. 2016;128(22):2830-2830.
- Kojima K, Konopleva M, Samudio IJ, et al. MDM2 antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy. *Blood*. 2005; 106(9):3150-3159.
- Reis B, Jukofsky L, Chen G, et al. Acute myeloid leukemia patients' clinical response to idasanutin (RG7388) is associated with pre-treatment MDM2 protein expression in leukemic blasts. *Haematologica*. 2016;101(5):e185-188.
- Holzer P, Masuya K, Furet P, et al. Discovery of a dihydroisoquinolinone derivative (NVP-CGM097): a highly potent and selective MDM2 inhibitor undergoing phase 1 clinical trials in p53wt tumors. *J Med Chem*. 2015;58(16):6348-6358.
- Furet P, Masuya K, Kallen J, et al. Discovery of a novel class of highly potent inhibitors of the p53-MDM2 interaction by structure-based design starting from a conformational argument. *Bioorg Med Chem Lett*. 2016; 26(19):4837-4841.
- Takahashi S. Downstream molecular pathways of FLT3 in the pathogenesis of acute myeloid leukemia: biology and therapeutic implications. *J Hematol Oncol*. 2011;4:13.
- Spiekermann K, Bagrintseva K, Schwab R, Schmieja K, Hiddemann W. Overexpression and constitutive activation of FLT3 induces STAT5 activation in primary acute myeloid leukemia blast cells. *Clin Cancer Res*. 2003; 9(6):2140-2150.
- Yoshimoto G, Miyamoto T, Jabbarzadeh-Tabrizi S, et al. FLT3-ITD up-regulates MCL-1 to promote survival of stem cells in acute myeloid leukemia via FLT3-ITD-specific STAT5 activation. *Blood*. 2009; 114(24):5034-5043.
- Papaemmanuil E, Gerstung M, Bullinger L, et al. Genomic classification and prognosis in acute myeloid leukemia. *N Engl J Med*. 2016;374(23):2209-2221.
- Seipel K, Marques MT, Bozzini M-A, Meinken C, Mueller BU, Pabst T. Inactivation of the p53-KLF4-CEBPA axis in acute myeloid leukemia. *Clin Cancer Res*. 2016;22(3):746-756.
- Lehmann C, Friess T, Birzele F, Kiialainen A, Dangl M. Superior anti-tumor activity of the MDM2 antagonist idasanutin and the Bcl-2 inhibitor venetoclax in p53 wild-type acute myeloid leukemia models. *J Hematol Oncol*. 2016;9(1):50.
- Weisberg E, Halilovic E, Cooke VG, et al. Inhibition of wild-type p53-expressing AML by the novel small molecule HDM2 inhibitor CGM097. *Mol Cancer Ther*. 2015; 14(10):2249-2259.
- Seipel K, Marques MAT, Sidler C, Mueller BU, Pabst T. The cellular p53 inhibitor MDM2 and the growth factor receptor FLT3 as biomarkers for treatment responses to the MDM2-inhibitor idasanutin and the MEK1 inhibitor cobimetinib in acute myeloid leukemia. *Cancers*. 2018;10(6).
- Swords R, Freeman C, Giles F. Targeting the FMS-like tyrosine kinase 3 in acute myeloid leukemia. *Leukemia*. 2012; 26(10):2176-2185.
- Kampa-Schittenhelm KM, Heinrich MC, Akmut F, Döhner H, Döhner K, Schittenhelm MM. Quizartinib (AC220) is a potent second generation class III tyrosine kinase inhibitor that displays a distinct inhibition profile against mutant-FLT3, -PDGFRA and -KIT isoforms. *Mol Cancer*. 2013;12:19.
- Weisberg E, Sattler M, Manley PW, Griffin JD. Spotlight on midostaurin in the treatment of FLT3-mutated acute myeloid leukemia and systemic mastocytosis: design, development, and potential place in therapy. *OncoTargets Ther*. 2017; 11:175-182.
- Foss B, Ulvestad E, Bruserud Ø. Platelet-derived growth factor (PDGF) in human acute myelogenous leukemia: PDGF receptor expression, endogenous PDGF release and responsiveness to exogenous PDGF isoforms by in vitro cultured acute myelogenous leukemia blasts. *Eur J Haematol*. 2001;67(4):267-278.
- Padró T, Bieker R, Ruiz S, et al. Overexpression of vascular endothelial growth factor (VEGF) and its cellular receptor KDR (VEGFR-2) in the bone marrow of patients with acute myeloid leukemia. *Leukemia*. 2002;16(7):1302-1310.
- Dos Santos C, McDonald T, Ho YW, et al. The Src and c-Kit kinase inhibitor dasatinib enhances p53-mediated targeting of human acute myeloid leukemia stem cells by chemotherapeutic agents. *Blood*. 2013; 122(11):1900-1913.
- Lei H, Velez G, Kazlauskas A. Pathological signaling via platelet-derived growth factor receptor (alpha) involves chronic activation of Akt and suppression of p53. *Mol Cell Biol*. 2011;31(9):1788-1799.
- Kent D, Copley M, Benz C, Dykstra B, Bowie M, Eaves C. Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway. *Clin Cancer Res*. 2008;14(7):1926-1930.
- Stubbs MC, Kim YM, Krivtsov AV, et al. MLL-AF9 and FLT3 cooperation in acute myelogenous leukemia: development of a model for rapid therapeutic assessment. *Leukemia*. 2008;22(1):66-77.
- Armstrong SA, Kung AL, Mabon ME, et al. Inhibition of FLT3 in MLL. Validation of a

- therapeutic target identified by gene expression based classification. *Cancer Cell*. 2003;3(2):173-183.
31. Stam RW, den Boer ML, Schneider P, et al. Targeting FLT3 in primary MLL-gene-rearranged infant acute lymphoblastic leukemia. *Blood*. 2005;106(7):2484-2490.
  32. Wattel E, Preudhomme C, Hecquet B, et al. p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. *Blood*. 1994; 84(9):3148-3157.
  33. Long J, Parkin B, Ouillette P, et al. Multiple distinct molecular mechanisms influence sensitivity and resistance to MDM2 inhibitors in adult acute myelogenous leukemia. *Blood*. 2010;116(1):71-80.
  34. Muller PAJ, Vousden KH. Mutant p53 in cancer: new functions and therapeutic opportunities. *Cancer Cell*. 2014; 25(3):304-317.
  35. Zauli G, Celeghini C, Melloni E, et al. The sorafenib plus nutlin-3 combination promotes synergistic cytotoxicity in acute myeloid leukemic cells irrespectively of FLT3 and p53 status. *Haematologica*. 2012; 97(11):1722-1730.
  36. Grisendi S, Mecucci C, Falini B, Pandolfi PP. Nucleophosmin and cancer. *Nat Rev Cancer*. 2006;6(7):493-505.
  37. Colombo E, Marine J-C, Danovi D, Falini B, Pelicci PG. Nucleophosmin regulates the stability and transcriptional activity of p53. *Nat Cell Biol*. 2002;4(7):529-533.
  38. Schlenk RF, Döhner K, Krauter J, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med*. 2008;358(18):1909-1918.
  39. Sperr WR, Zach O, Pöll I, et al. Karyotype plus NPM1 mutation status defines a group of elderly patients with AML ( $\geq 60$  years) who benefit from intensive post-induction consolidation therapy. *Am J Hematol*. 2016;91(12):1239-1245.
  40. Stone RM, Mandrekar S, Sanford BL, et al. The multi-kinase inhibitor midostaurin (M) prolongs survival compared with placebo (P) in combination with daunorubicin (D)/cytarabine (C) induction (ind), high-dose C consolidation (consol), and as maintenance (maint) therapy in newly diagnosed acute myeloid leukemia (AML) patients (pts) age 18-60 with FLT3 mutations (mut): an international prospective randomized (rand) P-controlled double-blind trial (CALGB 10603/RATIFY [Alliance]). *Blood*. 2015;126(23):6-6.
  41. Starr P. Midostaurin the first targeted therapy to improve survival in AML: potentially practice-changing. *Am Health Drug Benefits*. 2016;9(Spec Issue):1-21.
  42. Chou T-C. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res*. 2010; 70(2):440-446.