약학석사학위논문

Synergistic apoptosis induced by a targeted combination treatment with midostaurin and ABT199 in the FMS-like tyrosine kinase (FLT)3-internal tandem duplication (ITD)-positive and BCL2-overexpressing cell line MV4-11

MV4-11 (FMS-like tyrosine kinase (FLT)3-internal tandem duplication (ITD) 변이 및 BCL2 과발현) 세포주에서 midostaruin과 ABT199에 의한 표적 항암 치료

2019년 2월

서울대학교 대학원

약학과 의약생명과학전공

이소미

ABSTRACT

Synergistic apoptosis induced by a targeted combination treatment with midostaurin and ABT199 in the FMS-like tyrosine kinase (FLT)3-internal tandem duplication (ITD)-positive and BCL2-overexpressing cell line MV4-11

Somi Lee

College of Pharmacy

The Graduate School

Seoul National University

Acute myeloid leukemia (AML) is a heterogenous disease with low survival rate, and relapsed AML with FMS-like tyrosine kinase (FLT)3 **(**FLT3)-internal tandem duplication (ITD) mutations worsens the prognosis of the patients. After an initial profiling of AML cell lines, we selected the cell line MV4-11 presenting both the FLT3-ITD mutation as well as overexpression of the antiapoptotic BCL2 resistance protein. In an attempt to suggest a more efficient targeted chemotherapeutic approach, we determined sub-toxic doses of midostaurin targeting the FLT3-ITD and ABT199 (Venetoclax), a specific inhibitor of BCL2. Whereas subtoxic doses of both compounds did not strongly affect viability of MV4-11 cells, a combination treatment synergistically triggered caspase-dependent apoptotic cell death compared to AML cells lines without FLT3 mutation or cells with a healthy phenotype. Our results could

be validated by calculation of the combination index according to Chou-Talalay. Moreover, the cytotoxic effect of the combination treatment was evaluated by Annexin-PI staining and flow cytometry and detection of caspase activation with or without pre-treatment of the pan-caspase inhibitor z-VAD-FMK. Importantly, the combination of midostaurin and ABT199 reduced the tumor formation in *in vitro* colony formation assays and zebrafish xenografts. Altogether, we suggest a novel, more efficient chemotherapeutic approach for AML patients expressing both elevated levels of antiapoptotic BCL2 and mutated FLT3.

Key words: FLT3 mutation, BCL2 overexpression, acute myeloid leukemia, apoptosis, targeted therapy.

ABBREVIATIONS

AE:	**Adverse effect**
AML:	**Acute myeloid leukemia**
BCL2:	**B-cell leukemia/lymphoma 2**
BCL-xL:	**B-cell lymphoma extra large**
BH:	**BCL-2 Homology domain**
BSA:	**Bovine Serum Albumin**
CFA:	**Colony Formation Assay**
CLL:	**Chronic lymphocytic leukemia**
CR:	**Complete remission**
CML:	**Chronic myeloid leukemia**
DMSO:	**Dimethyl sulfoxide**
ERK:	**Extracellular signal-regulated kinases**
FDA:	**Food and Drug Administration**
FLT3:	**FMS-like Tyrosine kinase 3**
GI:	**Growth inhibition**

HPF:	**Hours Post Fertilization**
IC:	**Inhibitory concentration**
IDH:	**Isocitrate dehydrogenase**
ITD:	**Internal tandem duplication**
JAK:	**Janus kinase**
MAP:	**Mitogen-activated protein**
MPER:	**Mammalian Protein Extraction Reagent**
MTT:	**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide**
NHL:	**Non-Hodgkin's lymphoma**
ORR:	**Overall response rate**
PBS:	**Phosphate Buffered Saline**
PI:	**Propidium Iodide**
PI3K:	**Phosphatidylinositol 3 kinase**
PMSF:	**Phenylmethylsulphonyl fluoride**
RPMI:	**Roswell Institute Park Memorial Institute**
PTU:	**Phenylthiourea**
R/R:	**Relapsed/refractory**

RTK:	**Receptor tyrosine kinase**
SDS-PAGE:	**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis**
STAT:	**Signal transducer and activator of transcription**
WT:	**Wild type**

Table of Contents

1. INTRODUCTION

Acute myeloid leukemia (AML) is a hematological disorder of the myeloid cells. This disease is characterized by myeloid progenitor cells that lost in their ability to differentiate into mature blood cells. For over three decades, without any significant improvement, the 5-year overall survival rates for patients of 60 years and higher was ranging from 35% to 40% and the median overall survival was approximately one year [1]. Accordingly, research aiming at the discovery of novel therapeutic approaches whether single or combined treatments are urgently required.

In 2017, the first targeted therapy for AML was approved by the Food and Drug Administration (FDA) as a breakthrough treatment against AML with FMS-like tyrosine kinase 3-internal tandem duplication (FLT3-ITD) mutations [2]. The FLT3-ITD gene is found in about 30% of AML patients and approximately 75% of these patients present a poor prognosis with low survival and high relapse rates [3].

FMS-like tyrosine kinase 3 is a type III receptor tyrosine kinase (RTK) which is expressed in most acute leukemic as well as in normal hematopoietic cells. This receptor plays a key role in cell survival, proliferation, and differentiation. FLT3 mutations lead to constitutive activation of the receptor and subsequently trigger downstream signaling events including signal transducer and activator of transcription (STAT)-5, mitogen-activated protein (MAP) kinase and phosphatidylinositol 3 kinase/serine-threonine kinase (PI3K/AKT) activation

leading to suppression of apoptosis and activation of cell proliferation [4].

The staurosporine analogue, midostaurin is a clinically-used first-generation multi-kinase FLT3 inhibitor which has broad specificity against RTKs. Nevertheless, achievement of a complete remission (CR) was not observed when midostaurin was administered as a single agent [5]. Therefore, novel and more specific kinase inhibitors still need to be designed on one hand, but on the other, synergistic co-treatments could also contribute to improve therapeutic results. Recently, we investigated the effect of targeted anticancer agents against the antiapoptotic BCL2 protein family on survival and proliferation of leukemia cells. Among those agents, specific BCL2 inhibitor ABT-199 (Venetoclax, Venclexta) showed excellent anticancer activity alone or in combination with other experimental compounds [6, 7].

BCL2 family proteins are expressed by most cell types and are core regulators of cell survival and death. BCL2 family proteins are involved in both apoptotic and non-apoptotic cell death. A pathological imbalance of pro- and anti-apoptotic members of the BCL2 family was shown to promote cell survival in AML [8]. Recently, these proteins became important anticancer targets and ABT199 (Venetoclax) is an already clinically-used BCL2 inhibitor in chronic lymphocytic leukemia (CLL) [6].

In CLL, the overall response rate (ORR) was 48% and 79% in relapsed/refractory (R/R) CLL and non-Hodgkin's lymphoma (NHL), respectively. In AML, the clinical activity of ABT199 was less significant as

the ORR was 15.5% against R/R AML and severe adverse effects like anemia, neutropenia, pneumonia were observed. Interestingly, patients presenting a mutation of the isocitrate dehydrogenase (IDH)-1 gene were more sensitive to ABT199. To increase efficacy and reduce side effects, co-treatments of ABT-199 and other therapeutic agents were suggested. Indeed, 61.5% and 88% of R/R CML and NHL responded to ABT199 in combination with rituximab and bendamustine. Similar combination studies are ongoing in AML [9].

By using *in vitro* and *in vivo* assays, it was our aim to determine the synergistic effect of midostaurin and ABT199 in FLT3-mutated AML cells compared to FLT3 wild type (WT) or healthy human cells in order to provide a pre-clinical basis for a future targeted AML treatment.

2. MATERIALS AND METHODS

2.1 Compounds

Midostaurin (M1323, Sigma-Aldrich, USA) and ABT199 (GDC-0199, Selleckchem, USA) were used in a single and combination treatments. Etoposide (E1383, Sigma-Aldrich, USA) was used as the positive control, and caspase inhibitor 1 (z-VAD-FMK, 187389-52-2, Calbiochem, USA) served for the inhibition of caspase-dependent apoptosis.

2.2 Cell culture and treatments

Chronic myeloid leukemia (CML) cell line K562 and acute myeloid leukemia (AML) cell lines U937, MV4-11, MOLM13, and OCI-AML3 were purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSZM; Braunschweig, Germany). K562 and U937 cells were cultured in RPMI medium (Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum (FBS) (Biowest, Nuaille, France and Opti-Gold, GenDEPOT, USA) and 1% (v/v) antibiotic–antimycotic (Lonza, Basel, Switzerland) at 37 ℃ and 5% of CO_2. MV4-11, MOLM13, and OCI-AML3 were cultured in RPMI medium (Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum (FBS) (Opti-Gold, GenDEPOT, USA) and 1% (v/v) antibiotic–antimycotic (Lonza, Basel, Switzerland) at 37 ℃ and 5% of CO_2.

2.3 Cell Proliferation and viability

Cell proliferation and viability were measured by using the trypan blue exclusion assay (Lonza, Basel, Switzerland). The number of cells in 1 mL was counted, and the fraction of trypan blue-positive cells was estimated by using a Malassez cell counting chamber (Marienfeld, Lauda-Königshofen, Germany).

2.4 Protein extraction and western blots

Whole cell extracts were prepared using M-PER® (Thermofisher, Waltham, Massachusetts, USA) supplemented with 1x protease inhibitor cocktail (Complete EDTA-free, Roche, Basel, Switzerland) according to the manufacturer's instructions. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes (GE Healthcare, Little Chalfont, UK). Membranes were incubated with selected primary antibodies. Chemo-luminescence signal was detected with the ECL Select/Prime Western Blotting Detection System (GE Healthcare, Little Chalfont, UK) and quantified by ImageQuant LAS 4000 mini system (GE Healthcare).

2.5 Quantification of apoptosis

The percentage of apoptotic cells was quantified as the fraction of cells showing fragmented nuclei, as assessed by fluorescence microscopy (Nikon, Tokyo, Japan) after staining with Hoechst 33342 (Sigma-Aldrich, USA) and propidium

iodide (PI) (Sigma-Aldrich, USA). Apoptosis was also confirmed by Annexin V/PI staining and fluorescence-activated cell sorter (FACS) analysis according to the manufacturer protocol (BD Biosciences).

2.6 Colony formation assay

For colony formation assays, $1x10^3$ cells/mL were grown in a semi-solid methylcellulose medium (Methocult H4230, StemCell Technologies Inc., Vancouver, Canada) supplemented with 10% FBS and indicated concentrations. Colonies were detected after 10 days of culture by adding 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, USA) and were scored by Image J 1.8.0 software (U.S. National Institute of Health, Bethesda, MD, USA).

2.7 Investigation of cellular morphology

For Diff-Quik staining, cells were pretreated with midostaurin and ABT199 as single compounds or in combination at indicated concentrations. Cells were spun onto a glass slide for 5 minutes at 1500 rpm using a Cytopad with caps (Elitech biomedical systems, USA). Cells were then fixed and stained with the Diff-Quik staining kit (Dade Behring S.A., USA) according to the manufacture's protocol and pictures were taken (Nikon Eclipse Ti-U, Nikon, Japan).

2.8 Zebrafish xenografts

For xenograft assays, after mating, fertilized eggs were incubated in Danieau's solution with 0.003% of phenylthiourea (PTU) at 28 ℃ for 48 h [10]. Micropipettes for injection and anesthesia were generated from a 1.0 mm glass capillary (World Precision Instruments, FL, USA) by using a micropipette puller (Shutter Instrument, USA). 48 h post fertilization (hpf), zebrafish were anesthetized in 0.02 % tricaine (Sigma-Aldrich, USA) and immobilized on an agar plate. MV4-11 cells were treated with indicated compounds for 24 h; then cells were stained for additional 4 h with 4 μM of cell tracker CM-Dil dye (Invitrogen, USA). 100–200 of cells were injected into the yolk sac by microinjection (PV820 microinjector, World Precision Instruments, FL, USA). Subsequently, zebrafish were incubated in 24-well plates containing Danieau's solution with 0.003% phenylthiourea (PTU) at 28 ℃ for 72 h. Fish were then immobilized in a drop of 3% methylcellulose in Danieau's solution on a glass slide. Pictures were taken by fluorescence microscopy (Leica DE/DM 5000B, USA). Fluorescent tumors were quantified by Image J software (U.S. National Institute of Health, Bethesda, MD, USA).

2.9 Statistical analysis

Data are expressed as the mean ± s.d. and significance was estimated by using one-way or two-way ANOVA tests using Prism 7 software, GraphPad Software (La Jolla, CA, USA). P-values <0.05 were considered as significant. Combination index (CI) was calculated according to Chou and Talalay using Compusyn Software (ComboSyn, Inc., Paramus, NJ, USA). CI values < 1 indicate synergism.

3. RESULTS

3.1 Effect of midostaurin on the viability of AML cell lines with differential phosphorylation levels of FLT3 and BCL2 protein patterns

To investigate the effect of midostaurin on AML cells, we first characterized FLT3 protein expression and phosphorylation in different cell lines. Western blot analysis showed that MV4-11 AML cells expressed phosphorylated FLT3 at about 136 kD (double band). Other AML cell lines including U937, OCI-AML3 and chronic myeloid leukemia (CML) cell line K562 only weakly express WT FLT3 but are neither mutated, nor phosphorylated. MOLM13 cells are known to present a mutation in the FLT3 gene abrogating the expression of this protein [11] (Figure 1A).

In our previous studies, anti-apoptotic cell-type-specific expression of BCL2 family proteins notably MCL-1, BCL2 and BCL-xL were shown to contribute to cell survival whereas a targeted inhibition of these antiapoptotic regulators triggered apoptotic cell death in leukemia [6]. In Figure 1B, we used western blots to investigate the BCL2 family protein expression profile in each cell line. Interestingly, quantification of the western blot signal compared to actin expression levels allowed us to demonstrate that BCL2 is expressed in U937 cells but also FLT3-ITD mutated MV4-11 cells, compared to the other cell types (K562, MOLM13, OCI-AML3). In MV4-11, BCL2 appeared to be the most expressed BCL2-family protein, compared to BCL-xL and MCL-1 that is expressed at only very low levels.

In order to correlate the expression/phosphorylation levels of FLT3 to the sensitivity of the different cell models, we treated AML cells with increasing concentrations of midostaurin (Figure 1C). IC50s presented on Table I show a reduced sensitivity of U937, MOLM13 and OCI-AML3 compared to MV4-11 cells where midostaurin triggered a significant cytotoxic effect with an IC50 of 330.30 ± 44.45 nM. We also assessed the effect of midostaurin on proliferation of AML cell lines (Figure1D). Table II shows the cytostatic effect of midostaurin with a GI50 for MV4-11 of 37.25 ± 1.93 nM.

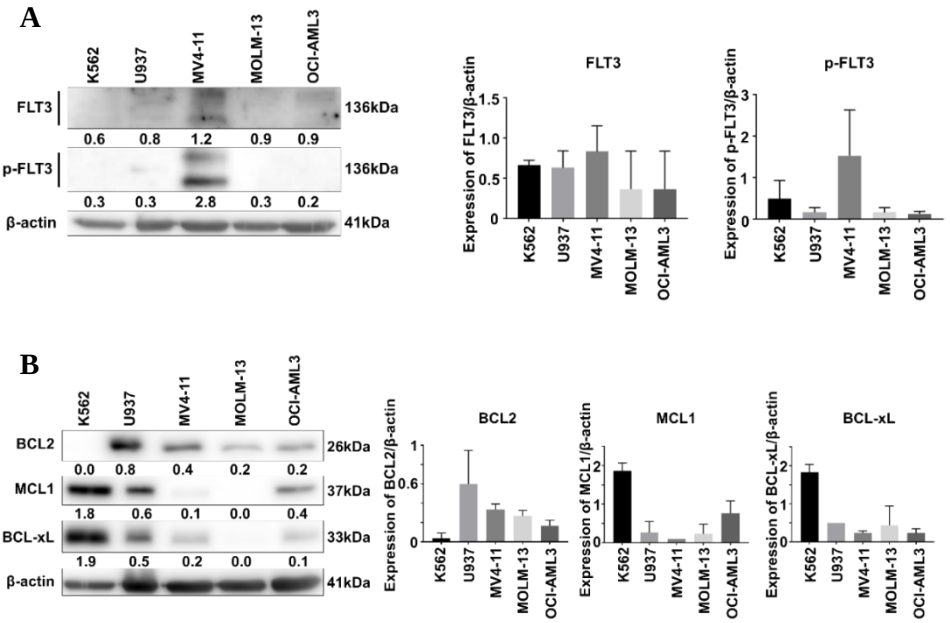
Based on the previously determined IC50 obtained with ABT199 for MV4-11 (130 nM), and in preparation of suitable concentrations for co-treatments of ABT199 and midostaurin, we further investigated a range of sub-toxic concentrations of ABT199 on MV4-11 at 8, 24, 48, 72 hours (Figure 1E). From this investigation, we selected the concentration of 10 nM as a suitable nontoxic concentration.

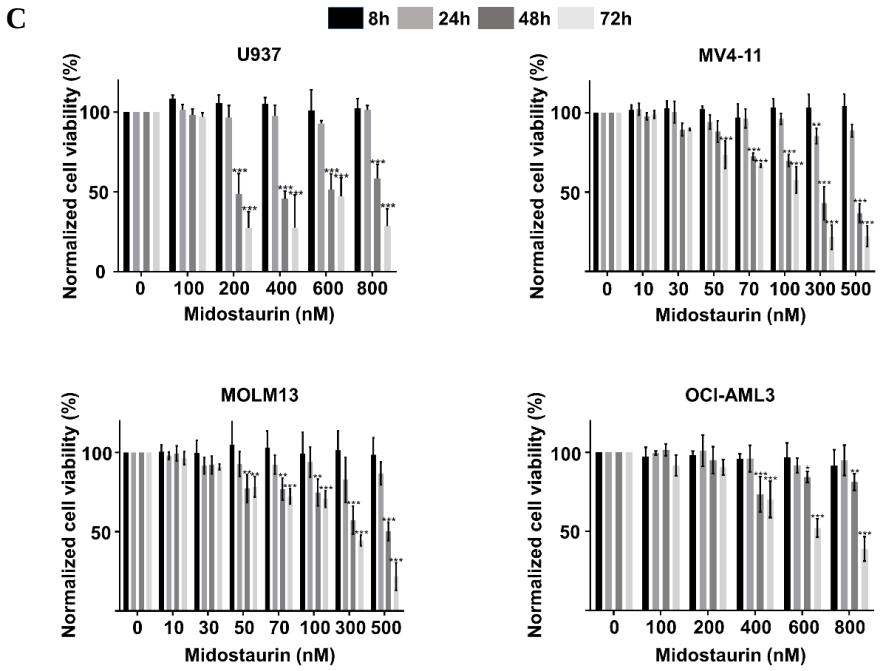
Subsequently, we also assessed the effect of midostaurin in colony formation assays. ABT199 showed a dose-dependent inhibition of colony formation with an IC50 of 71.88 ± 104.60 nM. Midostaurin also dose-dependently inhibited colony formation with an IC50 of 26.27 ± 28.69 nM (Figure 1F, Tables 3 and 4).

From the molecular point of view, we then assessed the effect of midostaurin of the FLT3 phosphorylation levels in MV4-11 cells. As expected, the phosphorylation of FLT3 was rapidly decreased by midostaurin at 30 nM, as

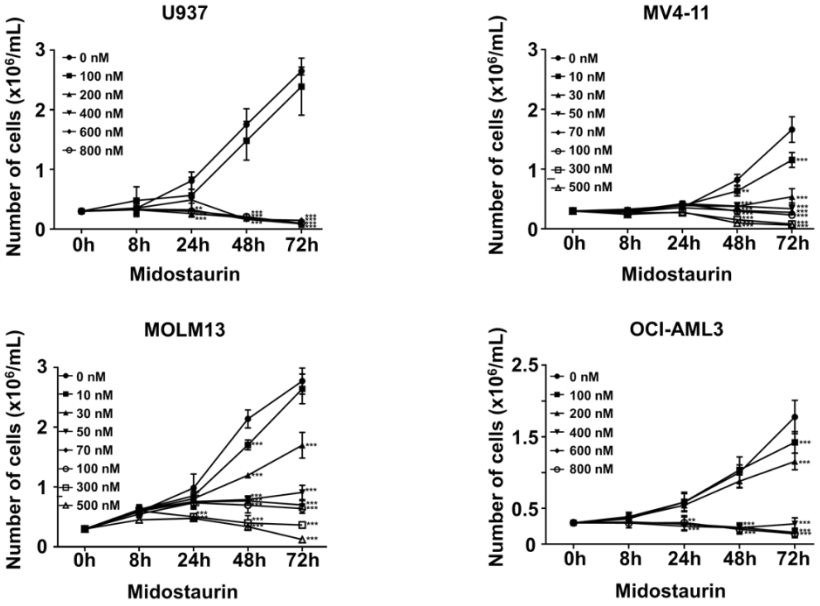
early as 5 minutes of treatment (Figure 1G). Moreover, the phosphorylation of FLT3 remained reduced over an 8-hour time course.

Altogether, our results demonstrated MV4-11 to be an interesting cell model presenting sensitivity towards midostaurin as well as ABT-199 due to the specific expression of the corresponding molecular targets as single treatments, at low nanomolar concentrations that are compatible with a pharmacological use.

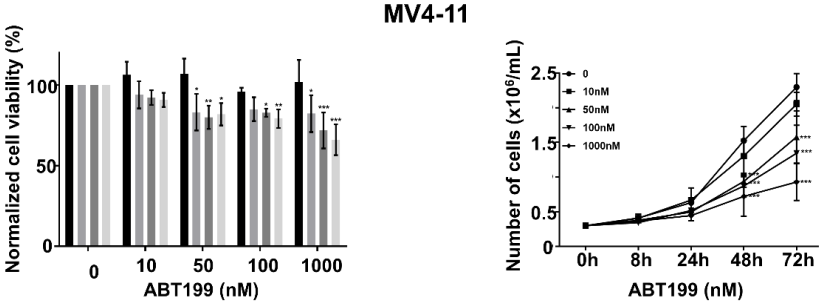

A
K562
U937
MV4-11
MOLM-13
OCI-AML3
FLT3
136kDa
0.6 0.8 1.2 0.9 0.9
p-FLT3
136kDa
0.3 0.3 2.8 0.3 0.2
β-actin
41kDa
FLT3
Expression of FLT3/β-actin
p-FLT3
Expression of p-FLT3/β-actin
B
BCL2
26kDa
0.0 0.8 0.4 0.2 0.2
MCL1
37kDa
1.8 0.6 0.1 0.0 0.4
BCL-xL
33kDa
1.9 0.5 0.2 0.0 0.1
β-actin
41kDa
BCL2
Expression of BCL2/β-actin
MCL1
Expression of MCL1/β-actin
BCL-xL
Expression of BCL-xL/β-actin

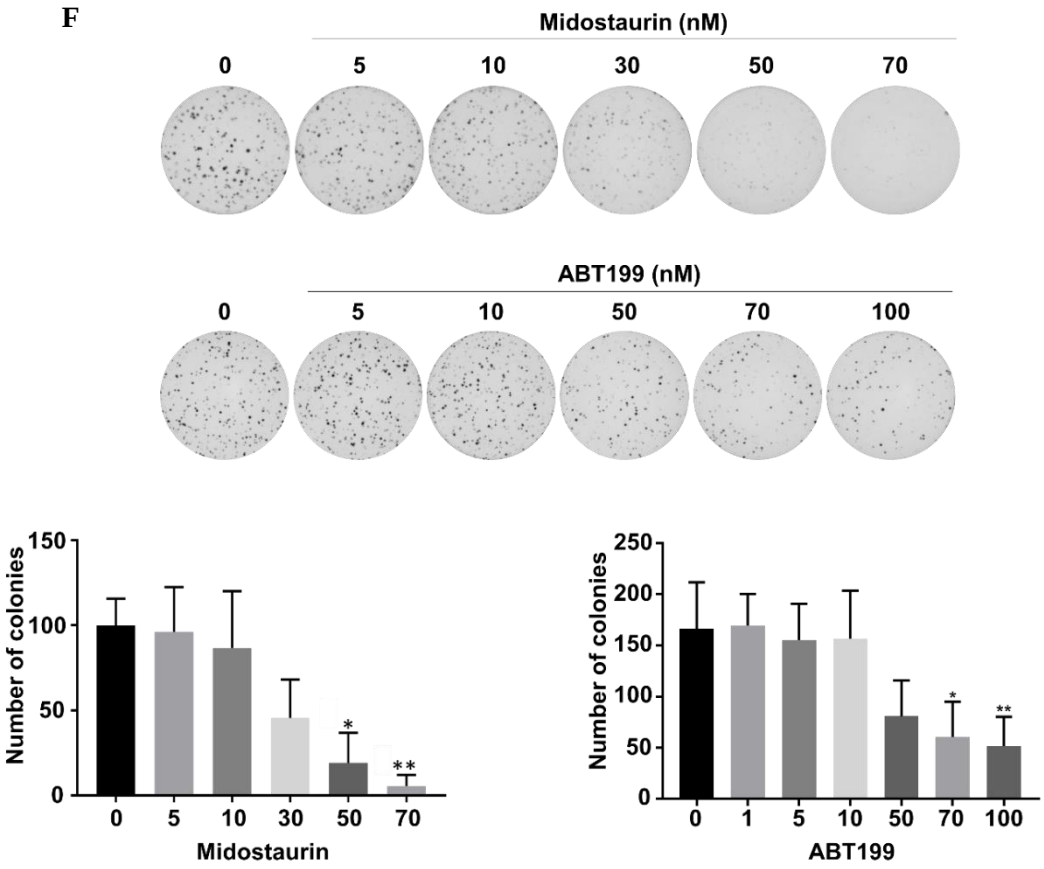

C
8h
24h
48h
72h
U937
MV4-11
MOLM13
OCI-AML3
Normalized cell viability (%)
Midostaurin (nM)
0 100 200 400 600 800
0 10 30 50 70 100 300 500

D


U937
Number of cells (x10⁶/mL)
0 nM
100 nM
200 nM
400 nM
600 nM
800 nM
0h 8h 24h 48h 72h
Midostaurin
MV4-11
Number of cells (x10⁶/mL)
0 nM
10 nM
30 nM
50 nM
70 nM
100 nM
300 nM
500 nM
0h 8h 24h 48h 72h
Midostaurin
MOLM13
Number of cells (x10⁶/mL)
0 nM
10 nM
30 nM
50 nM
70 nM
100 nM
300 nM
500 nM
0h 8h 24h 48h 72h
Midostaurin
OCI-AML3
Number of cells (x10⁶/mL)
0 nM
100 nM
200 nM
400 nM
600 nM
800 nM
0h 8h 24h 48h 72h
Midostaurin

E


Normalized cell viability (%)
0 10 50 100 1000
ABT199 (nM)
MV4-11
Number of cells (x10⁶/mL)
0
10nM
50nM
100nM
1000nM
0h 8h 24h 48h 72h
ABT199 (nM)



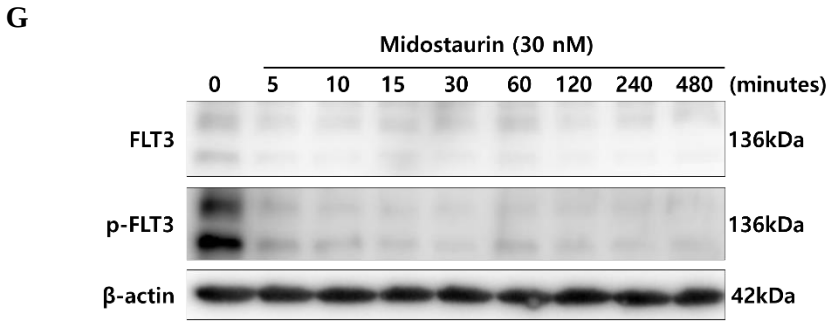


Figure 1. Effect of midostaurin on the viability of AML cell lines with

differential phosphorylation levels of FLT3 and BCL2 protein patterns. (A) Expression of FLT3 in AML cells and auto-phosphorylation of FLT3-ITD mutated MV4-11 cells. Chronic myeloid leukemia (CML) K562 cells were used for comparative studies (β-actin ratios). Quantification of the signals by Prism. Results correspond to at least three independent western blots. (B) Expression patterns of major anti-apoptotic Bcl-2 proteins in AML cell lines. K562 cells were used for comparative studies (β-actin ratios). Quantification of the signals by Prism. Results correspond to at least three independent western blots. (C) Cytotoxic effect and (D) anti-proliferative effect of midostaurin on AML cells after 24 h incubation, determined by trypan blue assay. (E) Effects of subtoxic concentrations of ABT199 on MV4-11 viability and proliferation. (F) Inhibitory effect of increasing concentrations of midostaurin and ABT199 on colony formation capacity. (G) Inhibition of phosphorylation of FLT3 in MV4-11 cells treated with 30nM of midostaurin.

Results correspond to the mean ± SD of three independent experiments; *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

Table 1: IC50 values determined in different AML cell lines treated with midostaurin by trypan blue assay

Cell line	IC50 (in nM, 48h)
U937	738.16±192.44
MV4-11	330.30±44.45
MOLM-13	452.66±84.97
OCI-AML3	>800

Table 2: GI50 values determined in different AML cell lines treated with midostaurin by trypan blue assay

Cell line	GI50 (in nM, 48h)
U937	136.90±3.00
MV4-11	37.25±1.93
MOLM-13	38.01±3.09
OCI-AML3	331.3±2.85

Table 3. Effect of midostaurin single treatment on colony formation.

Compound	Concentration (nM)	Count	Total Area	Average Size
Control	0	150.67±23.63	3033.67±642.36	20.14±2.54
Midostaurin	5	145.00±39.40	2280.33±346.93	16.43±4.49
	10	130.67±50.14	1669.33±307.52	14.43±4.72
	30	68.67±34.02	500.67±268.31	7.11±0.90
	50	29.00±26.46	109.33±85.45	4.45±1.93
	70	8.33±10.12	20.33±26.63	2.13±0.55

Table 4. Effect of ABT199 single treatment on colony formation.

Compound	Concentration (nM)	Count	Total Area	Average Size
Control	0	166.67±45.00	2102.67±302.72	13.48±4.94
ABT199	5	155.55±35.30	1812.00±97.04	12.16±3.32
	10	156.67±47.08	1488.33±187.37	9.90±2.33
	50	80.67±35.12	880.33±343.64	11.59±3.76
	70	60.67±34.31	566.00±238.87	10.20±2.39
	100	51.67±28.57	468.00±260.86	9.08±2.73

3.2 Midostaurin and ABT199 induce caspase-dependent apoptosis

In a next step we decided to characterize and quantify the type of cell death induced by midostaurin and ABT199 as single agents.

Apoptotic characteristics such as nuclear condensation and fragmentation were quantified by fluorescence microscopy after Hoechst/PI staining. Midostaurin (Figure 2A) and ABT199 (Figure 2B) dose-dependently induced apoptosis as single agents. Midostaurin induced significant levels of cell death at concentrations of 50 nM and above, whereas ABT-199 killed MV4-11 cells starting at a concentration of 5 nM. As the pan-caspase inhibitor zVAD-FMK (50 µM) was able to significantly protect cells against the effect of midostaurin or ABT199, we concluded that both agents trigger caspase-dependent apoptosis as single agents.

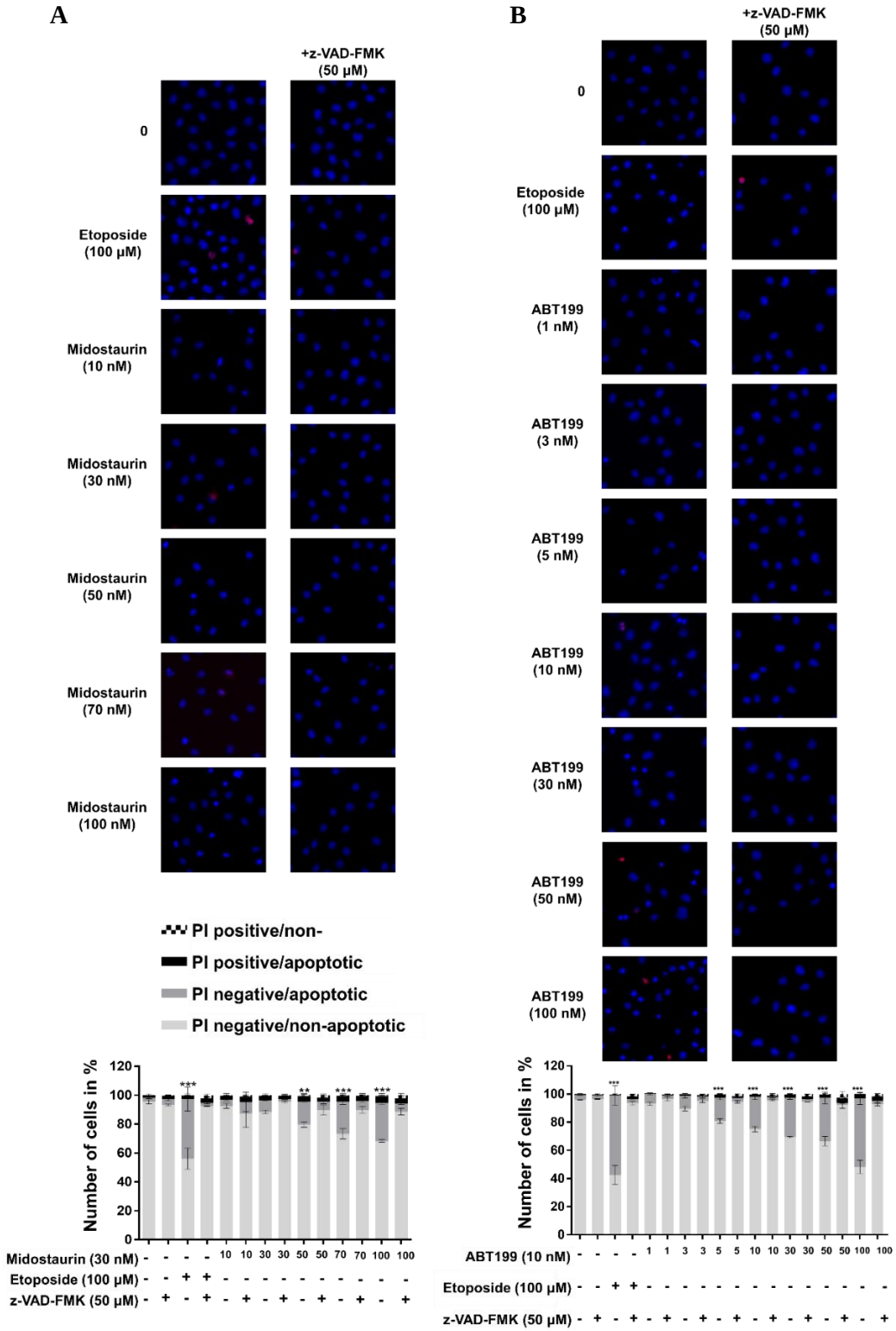

A
+z-VAD-FMK (50 µM)
0
Etoposide (100 µM)
Midostaurin (10 nM)
Midostaurin (30 nM)
Midostaurin (50 nM)
Midostaurin (70 nM)
Midostaurin (100 nM)
PI positive/non-
PI positive/apoptotic
PI negative/apoptotic
PI negative/non-apoptotic
Number of cells in %
Midostaurin (30 nM)
Etoposide (100 µM)
z-VAD-FMK (50 µM)
B
+z-VAD-FMK (50 µM)
0
Etoposide (100 µM)
ABT199 (1 nM)
ABT199 (3 nM)
ABT199 (5 nM)
ABT199 (10 nM)
ABT199 (30 nM)
ABT199 (50 nM)
ABT199 (100 nM)
Number of cells in %
ABT199 (10 nM)
Etoposide (100 µM)
z-VAD-FMK (50 µM)

Figure 2. Caspase-dependent apoptosis induced by single treatments of midostaurin and ABT199 in MV4-11 by nuclear morphology. Effect of (A) midostaurin and (B) ABT199 after 24 h incubation. Quantification of apoptotic cell by nuclear morphology.

Results correspond to the mean ± SD of three independent experiments; *p < 0.05, **p < 0.01, ***p <0.005.

3.3 Synergistic apoptotic effect of a combination of midostaurin and ABT199

Considering the pro-apoptotic effect of midostaurin and ABT199 as single agents, we then combined the two compounds to assess potential additive or synergistic effects. In order to assess the significance of a combination treatment, we used MV4-11 cells expressing the FLT3-ITD mutation as well as BCL2.

We then assessed the combination index (CI) according to Chou-Talalay by using data obtained from Hoechst/PI staining after 24 hours (Figure 3A). Midostaurin at 10, 30, 50, and 70 nM was combined to ABT199 at 1, 5, and 10 nM. Our results showed that all combinations generated a CI < 1 as indicated in Table 5. Based on the CI calculation, the combination of midostaurin at 30 nM and ABT199 at 10 nM reached CI of 0.27 witnessing a strong synergistic effect. We represented the results of the combination assays as a combination index plot (Figure 3B) based on the effect and synergies obtained with the various concentrations combinations of ABT199 and midostaurin.

We then analyzed the type of cell death that was induced by the combination treatment by annexin V/PI staining (Figure 3C). After 24 hours of treatment, the combination treatment increased cell death 1.7-fold compared to ABT199 alone and 2.9-fold compared to midostaurin alone (Figure 3D), whereas single treatments did not show a significant reduction of viable cells at these subtoxic concentrations by annexin V/PI staining. Furthermore, pretreatment with the

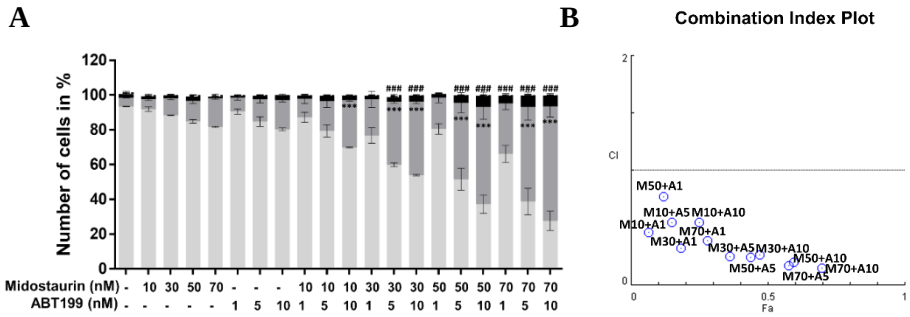
pan-caspase inhibitor, z-VAD-FMK, noticeably reduced the number of apoptotic cells in the combination treatment by 48.2% (Figure 3D). Altogether, combination treatments were able to induce synergistic, caspase-dependent cell death.

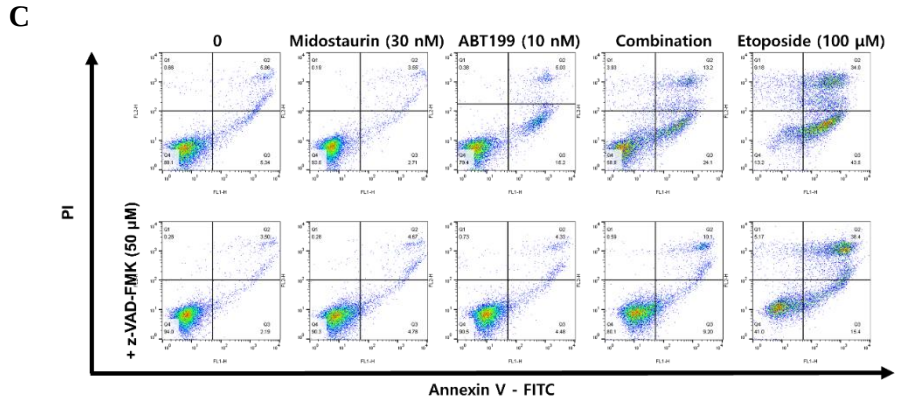
In an attempt to investigate molecular and cellular events that could cause cellular alterations leading to cell death, we visualized the effect of single and combination treatments using Diff-Quik staining of MV4-11 cells after 24 hours. Interestingly, we observe a 13.5-fold induction of cells developing the formation of vacuoles witnessing increased levels of cell stress in MV4-11 treated with sub-toxic concentrations of midostaurin (30 nM) and ABT199 (10 nM) after 24 hours (Figure 3E). As expected, in cells treated with a combination of both compounds, a dramatic increase of nuclear condensation, fragmentation and shrinkage of cytoplasm was observed.

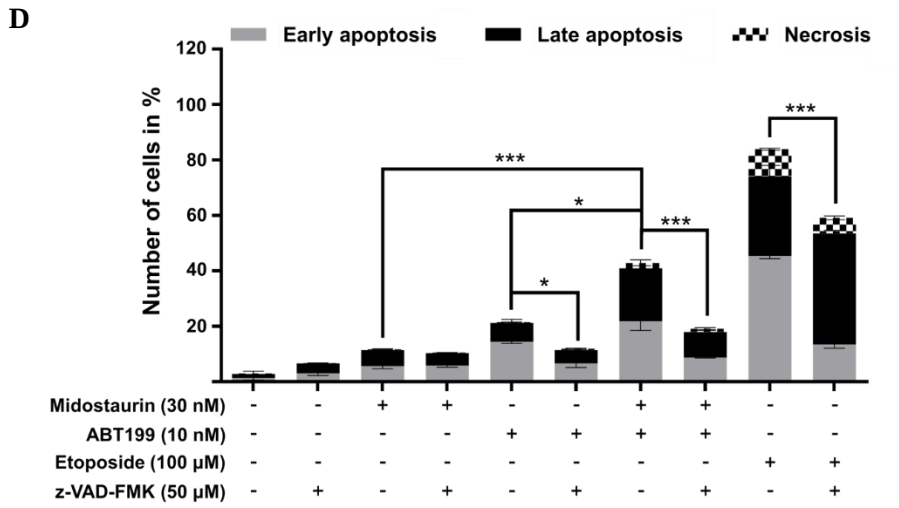
We then validated the differential toxicity of this combination treatment by assessing the cytotoxic and anti-proliferative effect on a B lymphocyte cell line (RPMI1788) presenting a healthy phenotype and the human epithelial L-132 cells after 8, 24, 48, and 72 hours (Figure 3F and G). Interestingly, RPMI1788 cells maintained their viability above 80% up to 72 hours after single or combination treatments (Figure 3F). The combination treatment induced only 6.6% of dead cells at 72 hours and proliferation was reduced by only 11.3% at 48 hours and by 11.2% at 72 hours. Moreover, viability and proliferation of L-132 cells was not affected by combination treatments (Figure 3G). Hence, we

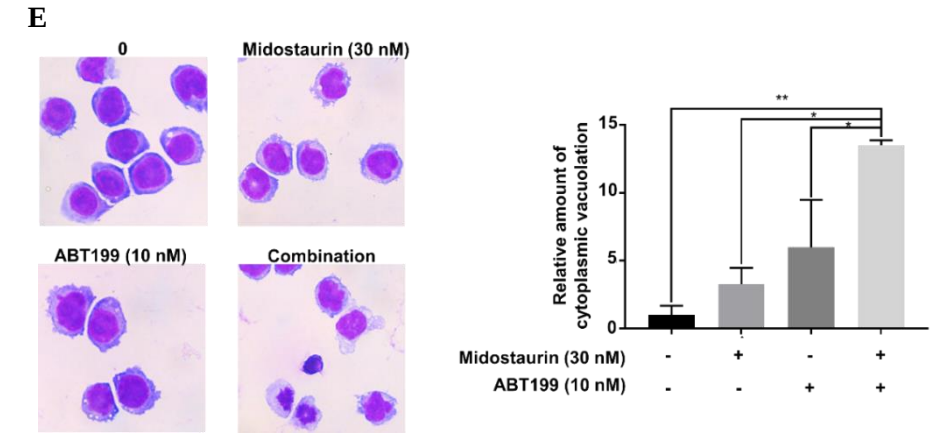
concluded that the combination treatment presents a specific effect on FLT3-ITD mutated AML cells expressing BCL2, without a significant effect on healthy cell models.

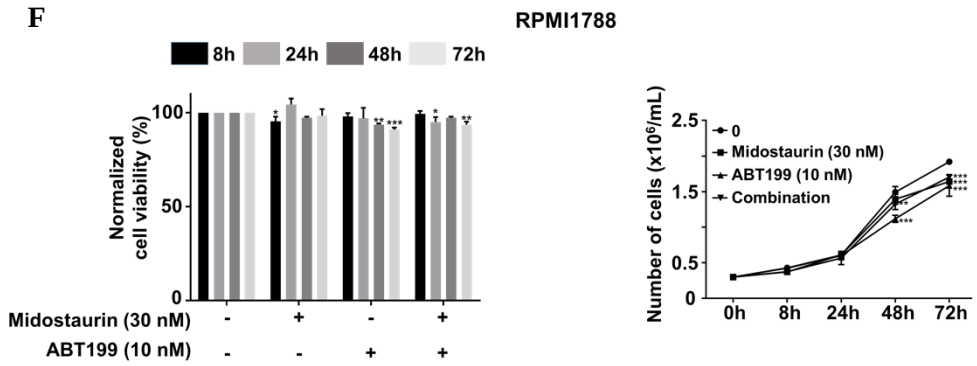
Moreover, we further explored the safety of combination treatments in the developing embryo of zebrafish where the heart rate and morphology remained unaffected, witnessing safety of this combination treatment in this preclinical setting (Figure 3H).

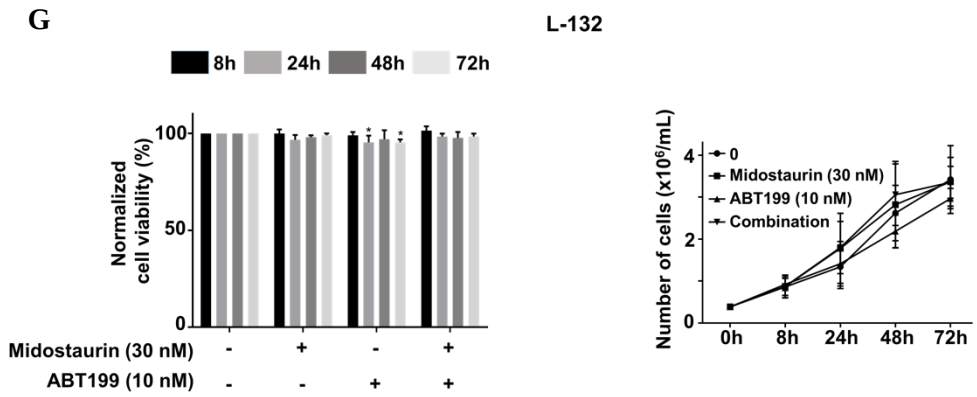

A
B
Combination Index Plot
Number of cells in %
Midostaurin (nM)
ABT199 (nM)
CI
Fa
M50+A1
M10+A5
M10+A10
M10+A1
M70+A1
M30+A1
M30+A5
M30+A10
M50+A10
M50+A5
M70+A10
M70+A5


C
0
Midostaurin (30 nM)
ABT199 (10 nM)
Combination
Etoposide (100 μM)
PI
+ z-VAD-FMK (50 μM)
Annexin V - FITC


D
Early apoptosis
Late apoptosis
Necrosis
Number of cells in %
Midostaurin (30 nM)
ABT199 (10 nM)
Etoposide (100 μM)
z-VAD-FMK (50 μM)


E
0
Midostaurin (30 nM)
ABT199 (10 nM)
Combination
Relative amount of cytoplasmic vacuolation
15
10
5
0
**
*
*
Midostaurin (30 nM) - + - +
ABT199 (10 nM) - - + +


F
RPMI1788
8h 24h 48h 72h
Normalized cell viability (%)
100
50
0
Midostaurin (30 nM) - + - +
ABT199 (10 nM) - - + +
Number of cells (x10^6/mL)
2.5
1.5
0.5
0
0h 8h 24h 48h 72h
0
Midostaurin (30 nM)
ABT199 (10 nM)
Combination


G
L-132
8h 24h 48h 72h
Normalized cell viability (%)
100
50
0
Midostaurin (30 nM) - + - +
ABT199 (10 nM) - - + +
Number of cells (x10^6/mL)
4
2
0
0h 8h 24h 48h 72h
0
Midostaurin (30 nM)
ABT199 (10 nM)
Combination

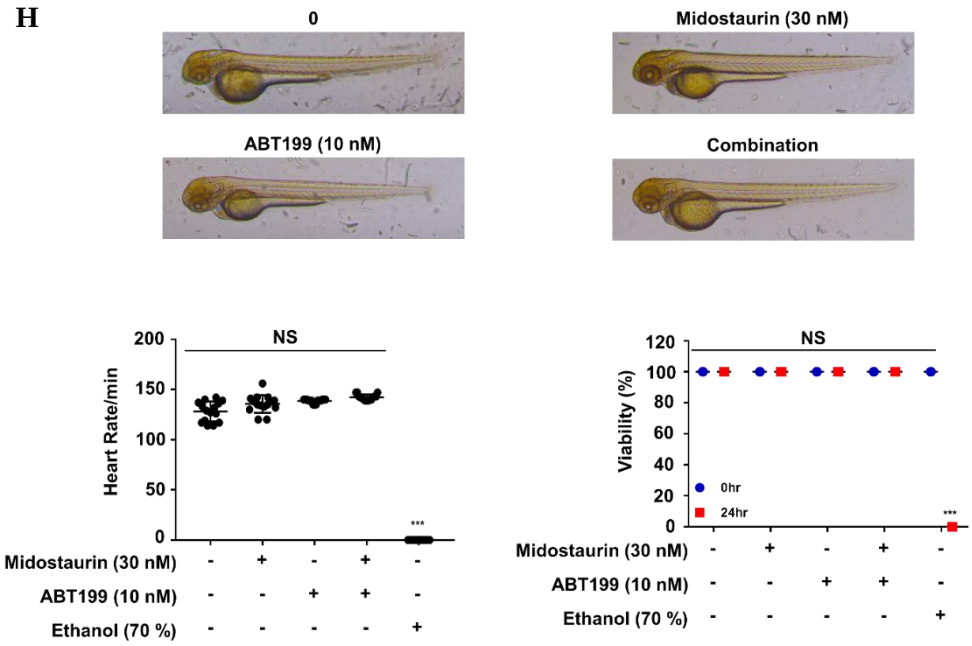


Figure 3. Synergistic apoptotic effect of a combination of midostaurin and ABT199. (A) Quantification of apoptotic cells in combination treatment (midostaurin 10, 30, 50, and 70nM with ABT199 1, 5, and 10nM) after 24 h by nuclear morphology. (B) Estimation of plots by combination index (CI) values (Y-axis) and at fraction affected (fa, X-axis) of combination treatment by Compusyn software (The ComboSyn, Inc.) (C) Effect of midostaurin and ABT199 single/combination treatments with/without z-VAD-FMK pre-treatment on MV4-11 cells by annexin-V/PI staining, and (D) quantification. (E) Observation of cellular morphology by single/combination treatment *via* Diff-Quik staining at 24 h. (F) Differential toxicity of midostaurin and ABT199 on healthy RPMI1788 and (G) L-132 cells. (H) *In vivo* toxicity assays on zebrafish by quantifying morphology, heart rate and viability.

Results correspond to the mean ± SD of three independent experiments; *p < 0.05, **p < 0.01, ***p <0.005.

Table 5: Calculation of the combination index values of midostaurin and ABT199 by Compusyn software.

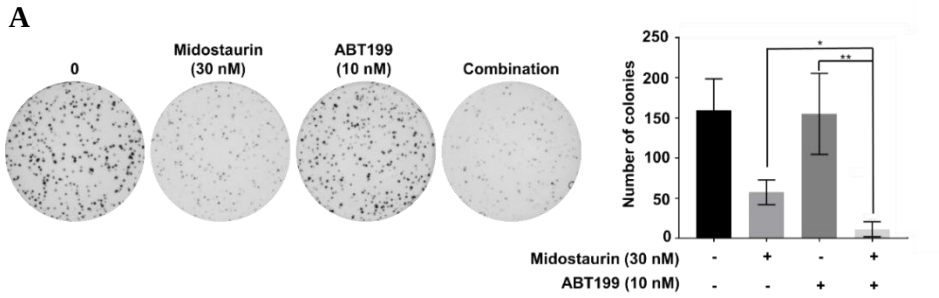
Midostaurin (nM)	ABT199 (nM)	Fa	CI	Midostaurin (nM)	ABT199 (nM)	Fa	CI
10	1	0.07	0.60	50	1	0.14	0.76
	5	0.15	0.57		5	0.45	0.22
	10	0.25	0.44		10	0.60	0.14
30	1	0.18	0.32	70	1	0.29	0.43
	5	0.36	0.25		5	0.58	0.17
	10	0.47	0.27		10	0.70	0.11

3.4 Validation of synergistic combination treatments by colony formation assays and *in vivo* zebrafish xenografts

After investigating the effect of midostaurin and ABT199 in suspension cultures, we aimed at demonstrating that the combination therapy was also effective in 3D cell cultures by colony formation assays (CFA) and *in vivo* zebrafish xenografts.

Combination of midostaurin (30nM) and ABT199 (10 nM) showed a significant reduction of the total number of colonies (Figure 4A), total area of colonies, and average size of colonies compared to untreated control and single treatments (midostaurin: 2.3-fold, ABT199: 7.4-fold) (Table 6).

We also extended our results by using an *in vivo* xenograft approach. The combination of midostaurin (30 nM) and ABT199 (10 nM) led to a complete abrogation of tumor formation in pretreated MV4-11 cells (Figure 4B).


A
0
Midostaurin (30 nM)
ABT199 (10 nM)
Combination
Number of colonies
250
200
150
100
50
0
*
**
Midostaurin (30 nM) - + - +
ABT199 (10 nM) - - + +

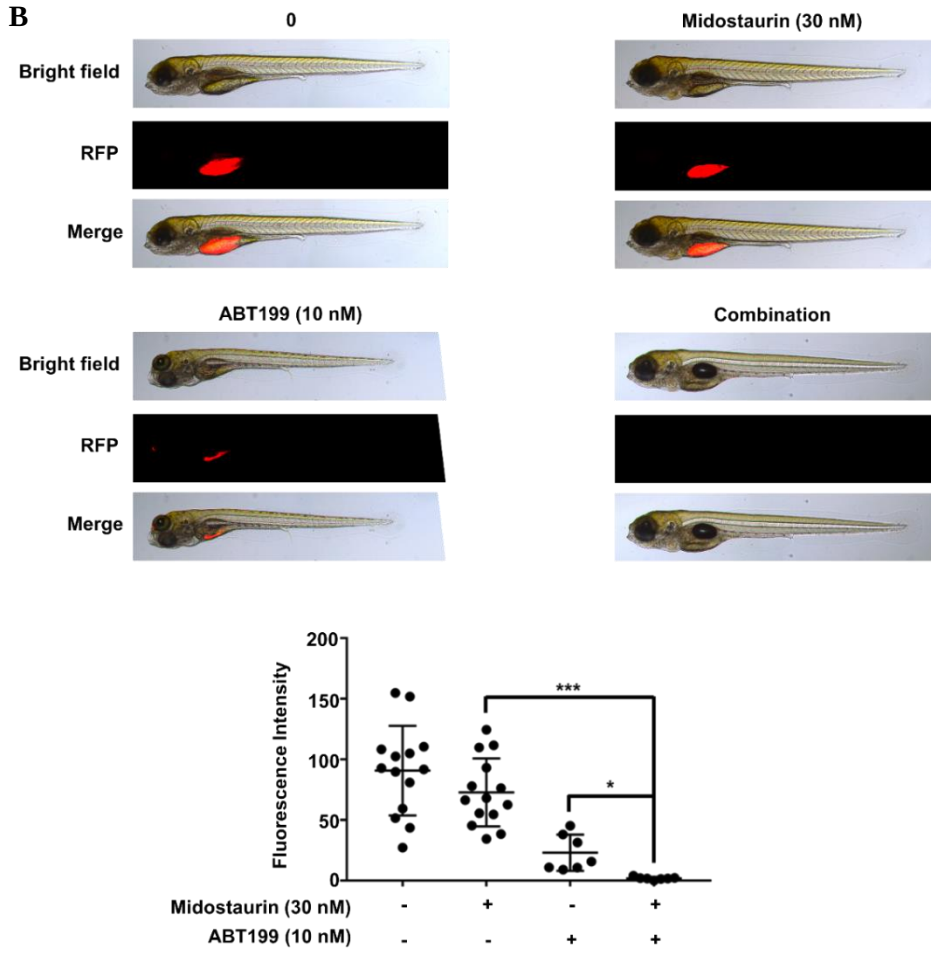

B
0
Midostaurin (30 nM)
Bright field
RFP
Merge
ABT199 (10 nM)
Combination
Bright field
RFP
Merge
Fluorescence Intensity
200
150
100
50
0
***
*
Midostaurin (30 nM) - + - +
ABT199 (10 nM) - - + +

Figure 4. Validation of synergistic combination treatments by colony formation assays and *in vivo* zebrafish xenografts. (A) Assessment of synergistic effect of the combination treatment on colony formation capacity. (B) *In vivo* anti-cancer effect on zebrafish xenografts by quantifying fluorescence.

Results correspond to the mean ± SD of three independent experiments; *p < 0.05, **p < 0.01, ***p <0.005.

Table 6. Effect of combination treatment by colony formation assay.

Compound	Concentration (nM)	Count	Total Area	Average Size
Control	0	159.33±39.27	2393.67±878.81	15.14±1.41
Midostaurin	30	92.67±54.68	827.00±844.23	7.96±4.31
ABT199	10	119.67±80.01	1609.67±1251.63	12.25±6.08
Midostaurin + ABT199	10 + 30	11.33±9.29	64.33±65.06	4.09±2.96

4. DISCUSSION

For this project, we investigated the effect of midostaurin combined to ABT199 on the FLT3-ITD expressing cell line MV4-11 by using *in vitro* and *in vivo* models. Our data showed that midostaurin combined to ABT199 induced a dose-dependent synergistic cytotoxicity in MV4-11.

With over 10.000 death in 2018 in the United States, AML will kill more patients than any other type of leukemia according to the National Cancer Institute Surveillance, Epidemiology and End Results (NCI SEER) program. Therefore, novel treatments or combinations are most urgently required. Treatments that target specific mutant proteins are most essential novel drugs undergoing clinical investigations in 2018. After 25 years without a novel drug against AML, two molecularly targeted drugs were approved and tested: midostaurin and enasidenib target the proteins encoded by two of the most commonly mutated genes in AML, FLT3 and IDH, respectively.

Midostaurin (Rydapt) was approved in April 2017. First-generation FLT3 inhibitors, including midostaurin, were clinically tested to treat AML patients expressing the FLT3-ITD mutation. FLT3 internal tandem duplications (ITDs) and point mutations occur in about 30% of all AML cases, and they tend to make the disease both more aggressive and more resistant to standard chemotherapy. Unfortunately, a single agent-based approach was not efficient for midostaurin [12]. Indeed, the first-generation FLT3 inhibitors affected a broad range of RTKs and generated toxic side effects associated with the

inhibitor's off-target effects [12]. These results limited the use of FLT3 inhibitors at relatively elevated concentrations.

Moreover, responses observed after inhibitor treatments were only temporary as resistance mechanisms were shown to overcome the effect of FLT3 inhibitors through mutations or induction of anti-apoptotic molecules including BCL2 or MCL-1 [12]. Therefore, targeting these BCL2-family proteins, that play an essential role in cell death resistance and proliferation, by a targeted drug may provide a synergistic effect at lower concentrations thus preventing off-target side effects [13, 14].

Hence, we suggested a strategy based in part on the BCL2 family protein expression levels. BCL2 family proteins are expressed by most cancer cell types but it is a cell-type specific expression pattern of one or several of the BCL2 family proteins that contributes to cell death resistance [13]. Indeed, the BCL2 family protein expression profile in AML patients varies among individuals. Profiling the patient followed by targeting the most overexpressed isoform could strongly sensitize the patient or cell line to the effect of other targeted inhibitors [6].

First, we demonstrated the overall cytotoxicity of each individual compound on AML cell lines. FLT3-mutated cell lines were the most sensitive towards midostaurin compared to the WT FLT3 cell lines. Interestingly, the BCL2 overexpressing FLT3 mutant cell line MV4-11 was significantly sensitive towards selective BCL2 inhibitor, ABT199. We maintained this unique cell

model to assess the effect of sub-toxic doses of midostaurin and ABT199 in combination trials [15].

There are several approaches to induce apoptotic cell death by combination treatments with BH3 mimetics such as ABT199, ABT263, or A-1210477. Interestingly, combinations of the above mentioned small molecule BH3 mimetics with other natural compounds successfully induced synergistic effects introducing benefits such as dose reduction and intensification of the effect. For example, our recent results showed that a combination with a BH3 mimetic transforms the cytostatic effect of selected coumarin derivatives into an immunogenic cytotoxic effect [7, 16]. On the other hand, dual treatment by BH3 mimetics, such as combination of a specific MCL-1 inhibitor and a targeted BCL2 inhibitor were effective in diverse AML subtypes [17]. Moreover, the sensitivity against ABT199 is greatly associated with the high expression levels of BCL2 and low expression of MCL-1 [18]. Thus, we hypothesized that the co-treatment of a BH3 mimetic may show synergistic effect with midostaurin.

We indeed demonstrated synergistic cytotoxic effects *in vitro via* various techniques including Diff-Quik, Hoechst/PI or Annexin V/PI staining. By fluorescent microscopy, we clearly showed nuclear shrinkage and fragmentation hinting at apoptotic cell death. The use of the pan-caspase inhibitor z-VAD-FMK allowed the conclusion that single and combination treatments induce caspase-dependent apoptosis. By using 3D culture system,

we also validated the synergistic effect of our combination treatment, in comparison to control, midostaurin, or ABT199 when used as a single agent. The effect of the combination treatment was validated by *in vivo* zebrafish xenografts showing that tumor formation in the zebrafish was completely abrogated. Altogether, our data clearly show the inhibitory effect of the combination treatment under consideration of tumor microenvironment.

Common adverse effects (AEs) in AML patients were nausea, diarrhea, fatigue, neutropenia, and vomiting and severe AEs as febrile neutropenia, anemia, and pneumonia were observed in phase II study [9]. Acceptable safety profile was evaluated. Disappointingly, ABT199 mono-therapy showed complete remission in only 6% and all (N=29) AML patients discontinued the treatment due to disease progression, AE such as terminal ileitis or patient withdrawal of consent, etc [19]. Interestingly, patients that were already pretreated with other therapeutic agents against AML showed a better response compared to untreated patients. First combination studies were started involving hypomethylating agents like cytarabine, azacytidine or decitabine [19].

We confirmed the safety of the combination treatment by testing our compounds on normal cells and zebrafish. We determined the differential toxicity on RPMI 1788 (normal lymphocytes) and L-132 (normal fibroblasts). Both single and combination treatments did not affect the normal cell's viability and proliferation. In our experiences, some cytotoxic compounds may affect the development, heart rate, and even viability of zebrafish, but here we

observed that our novel combination approach maintained all physiological functions in a non-toxic range.

For further research, we are planning to test the effect of co-treatment of midostaurin and ABT199 on the patient cells and patient derived xenografts (PDX).

Interestingly, the cell signaling pathways triggered by the FLT3 receptor activate a stress kinase pathway involving RAS, RAF and extracellular regulated kinase (ERK). It will be our perspective to investigate the effect of single and combined treatments on this pathway. Moreover, the Janus activated kinase (JAK) and STAT3/5 are under the control of FLT3. We intend to investigate the effect of FLT3 on these pathways specifically in AML cell lines and patient cells. Eventually, the PI3K/AKT pathway will be studied. We plan to investigate these pathways by both chemical and genetic inhibitors involving siRNA or short hairpin (sh) RNA mediated inhibition of these essential cell signaling intermediates. So far, our results indicated caspase-dependent induction of apoptosis by single or combined treatments. It will be our objective to investigate activation of the mitochondrial cell death pathway including cleavage of pro-caspases -3 and -9 as well as PARP-1 by western blot and luminescent caspase assays.

In conclusion, AML cells are very rapidly progressing and present a heterogeneous karyotype. In Figure 5, we suggest and overview our results obtained so far as well as our future molecular targets that remain to be

investigated. FLT3-ITD is becoming an interesting molecular target as the survival of patients with this mutation is particularly reduced. Hence, we suggest here a combination strategy based on the FLT3 mutational status and BCL2 protein expression profile as an attempt to improve the personalized treatment of such patients. Since midostaurin and ABT199 were already approved by the FDA, our combination treatments, at a low nanomolar range, could be rapidly clinically applied to improve the survival of patients with FLT3-mutated AML.

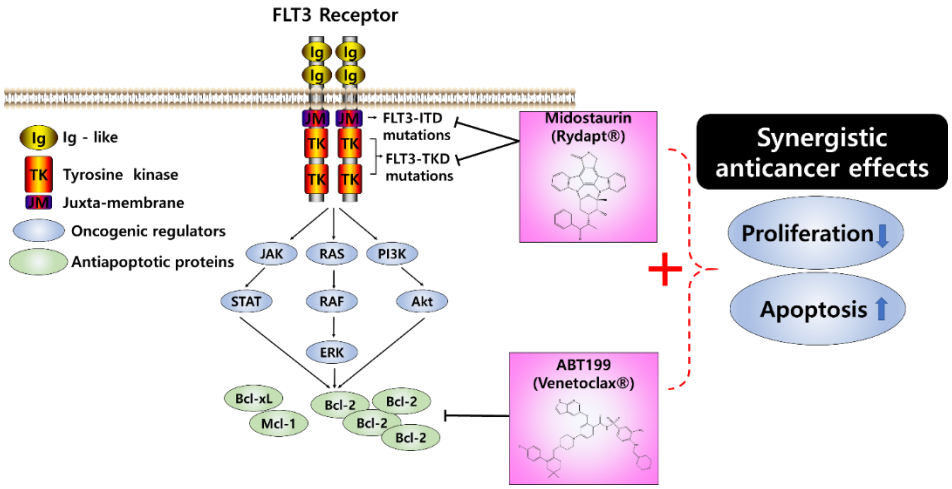


Figure 5: Schematic overview of the targeted interference with the FLT3 cell signaling pathway by FLT3-ITD inhibitor midostaurin and BCL2 inhibitor ABT199.

5. REFERENCES

[1] D. Thomas, R. Majeti, Biology and relevance of human acute myeloid leukemia stem cells, Blood, 129 (2017) 1577-1585.

[2] H. Wu, A. Wang, Z. Qi, X. Li, C. Chen, K. Yu, F. Zou, C. Hu, W. Wang, Z. Zhao, J. Wu, J. Liu, X. Liu, L. Wang, W. Wang, S. Zhang, R.M. Stone, I.A. Galinsky, J.D. Griffin, D. Weinstock, A. Christodoulou, H. Wang, Y. Shen, Z. Zhai, E.L. Weisberg, J. Liu, Q. Liu, Discovery of a highly potent FLT3 kinase inhibitor for FLT3-ITD-positive AML, Leukemia, 30 (2016) 2112-2116.

[3] D. Small, FLT3 mutations: biology and treatment, Hematology Am Soc Hematol Educ Program, 30 (2006) 178-184.

[4] Y. Furukawa, H.A. Vu, M. Akutsu, T. Odgerel, T. Izumi, S. Tsunoda, Y. Matsuo, K. Kirito, Y. Sato, H. Mano, Y. Kano, Divergent cytotoxic effects of PKC412 in combination with conventional antileukemic agents in FLT3 mutation-positive versus -negative leukemia cell lines, Leukemia, 21 (2007) 1005-1014.

[5] L.C. Stansfield, D.A. Pollyea, Midostaurin: A New Oral Agent Targeting FMS-Like Tyrosine Kinase 3-Mutant Acute Myeloid Leukemia, Pharmacotherapy, 37 (2017) 1586-1599.

[6] C. Cerella, A. Gaigneaux, A. Mazumder, J.Y. Lee, E. Saland, F. Radogna, T. Farge, F. Vergez, C. Recher, J.E. Sarry, K.W. Kim, H.Y. Shin, M. Dicato, M. Diederich, Bcl-2 protein family expression pattern determines synergistic pro-apoptotic effects of BH3 mimetics with hemisynthetic cardiac glycoside UNBS1450 in acute myeloid leukemia, Leukemia, 31 (2017) 755-759.

[7] J.Y. Lee, O. Talhi, D. Jang, C. Cerella, A. Gaigneaux, K.W. Kim, J.W. Lee, M. Dicato, K. Bachari, B.W. Han, A.M.S. Silva, B. Orlikova, M. Diederich, Cytostatic hydroxycoumarin OT52 induces ER/Golgi stress and STAT3 inhibition triggering non-canonical cell death and synergy with BH3 mimetics in lung cancer, Cancer Lett, 416 (2018) 94-108.

[8] M. Konopleva, A. Letai, BCL-2 inhibition in AML: an unexpected bonus?, Blood, 132 (2018) 1007-1012.

[9] S. Cang, C. Iragavarapu, J. Savooji, Y. Song, D. Liu, ABT-199 (venetoclax) and BCL-2 inhibitors in clinical development, J Hematol Oncol, 8 (2015) 129.

[10] J.Y. Lee, A. Mazumder, M. Diederich, Preclinical Assessment of the Bioactivity of the Anticancer Coumarin OT48 by Spheroids, Colony Formation Assays, and Zebrafish Xenografts, Journal of visualized experiments : JoVE, (2018).

[11] H. Quentmeier, J. Reinhardt, M. Zaborski, H.G. Drexler, FLT3 mutations in acute myeloid leukemia cell lines, Leukemia, 17 (2003) 120.

[12] M. Larrosa-Garcia, M.R. Baer, FLT3 Inhibitors in Acute Myeloid Leukemia: Current Status and Future Directions, Mol Cancer Ther, 16 (2017) 991-1001.

[13] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell, 144 (2011) 646-674.

[14] R. Swords, C. Freeman, F. Giles, Targeting the FMS-like tyrosine kinase 3 in acute myeloid leukemia, Leukemia, 26 (2012) 2176-2185.

[15] C.E. Annesley, P. Brown, The Biology and Targeting of FLT3 in Pediatric

Leukemia, Front Oncol, 4 (2014) 263.

[16] C. Cerella, F. Muller, A. Gaigneaux, F. Radogna, E. Viry, S. Chateauvieux, M. Dicato, M. Diederich, Early downregulation of Mcl-1 regulates apoptosis triggered by cardiac glycoside UNBS1450, Cell death & disease, 6 (2015) e1782.

[17] D.M. Moujalled, G. Pomilio, C. Ghiurau, A. Ivey, J. Salmon, S. Rijal, S. Macraild, L. Zhang, T.C. Teh, I.S. Tiong, P. Lan, M. Chanrion, A. Claperon, F. Rocchetti, A. Zichi, L. Kraus-Berthier, Y. Wang, E. Halilovic, E. Morris, F. Colland, D. Segal, D. Huang, A.W. Roberts, A.L. Maragno, G. Lessene, O. Geneste, A.H. Wei, Combining BH3-mimetics to target both BCL-2 and MCL1 has potent activity in pre-clinical models of acute myeloid leukemia, Leukemia, (2018).

[18] C. Touzeau, C. Dousset, S. Le Gouill, D. Sampath, J.D. Leverson, A.J. Souers, S. Maiga, M.C. Bene, P. Moreau, C. Pellat-Deceunynck, M. Amiot, The Bcl-2 specific BH3 mimetic ABT-199: a promising targeted therapy for t(11;14) multiple myeloma, Leukemia, 28 (2014) 210-212.

[19] M. Konopleva, D.A. Pollyea, J. Potluri, B. Chyla, L. Hogdal, T. Busman, E. McKeegan, A.H. Salem, M. Zhu, J.L. Ricker, W. Blum, C.D. DiNardo, T. Kadia, M. Dunbar, R. Kirby, N. Falotico, J. Leverson, R. Humerickhouse, M. Mabry, R. Stone, H. Kantarjian, A. Letai, Efficacy and Biological Correlates of Response in a Phase II Study of Venetoclax Monotherapy in Patients with Acute Myelogenous Leukemia, Cancer discovery, 6 (2016) 1106-1117.

6. 국문초록

급성 골수성 백혈병 (Acute myeloid leukemia, AML)은 골수성 혈액암으로서, 유전학적, 생리학적 이상으로 발병되는 것으로 보고되어 있다. 특히, FMS-like tyrosine kinase 3 (FLT3)는 세포 생존, 증식 및 분화에 핵심적인 역할을 하는 수용체로, 특히 재발환자에서 나타나는 FLT3-ITD 돌연변이는 그 예후를 악화한다.

본 연구에서는 단백질의 기초 발현 수준을 다양한 AML 세포주에서 확인하고 FLT3 인산화 및 Bcl-2 관련 단백질의 과발현 특성을 가진 MV4-11 세포주를 선택하였다.

보다 효율적인 표적 화학 요법을 제안하기 위해 FLT3-ITD 와 BCL-2 를 선택적으로 억제하는 midostaurin (Rydapt)과 ABT199 (Venetoclax)의 sub-toxic 한 농도를 산출하였다.

두 화합물의 Sub-toxic 한 농도는 각각 MV4-11 세포주에서 생존능력에 큰 영향을 미치지 않은 것과 대조적으로, 동반처리 후 caspase 의존성 세포사멸기전을 유도함으로써 유의미한 상승효과를 확인하였다. 뿐만 아니라, 이 조합은 정상 세포주에서 유의미한 세포독성을 나타내지 않았다. 이 결과를 바탕으로 통계적으로 Chou-Talalay 의 CompuSyn 소프트웨어를 이용하여 combination index 를 산출하였다.

또한, 두 조합에 의한 세포독성을 annexin V/PI 염색을 통해 flow cytometry 로 정량 하였고, 전체 caspase 를 억제하는 z-VAD-FMK 처리를 통해 Caspase 의존성 세포사멸 기전을 검증하였다.

뿐만 아니라, 3-dimensional system 인 colony formation assay 와 *in vivo* zebrafish xenografts 를 통한 종양억제효과를 분석함으로서 항암효과를 확인하였다. 결론적으로, Bcl-2 과발현 및 FLT-3 변이성 AML 환자의 개인 맞춤형 치료 전략에 midostaurin 과 ABT199 의 새로운 최적의 치료조합을 제안한다.

주요어: FLT3 변이, BCL2 과발현, 급성 골수성 백혈병, 세포사멸, 표적치료