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Improved efficacy of quizartinib in combination therapy with PI3K inhibition in primary FLT3-ITD AML cells

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ABSTRACT

Acute myeloid leukemia is a heterogeneous hematopoietic malignancy, characterized by uncontrolled clonal proliferation of abnormal myeloid progenitor cells, with poor outcomes.

The internal tandem duplication (ITD) mutation of the Fms-like receptor tyrosine kinase 3 (FLT3) (FLT3-ITD) represents the most common genetic alteration in AML, detected in approximately 30% of AML patients, and is associated with high leukemic burden and poor prognosis. Therefore, this kinase has been regarded as an attractive druggable target for the treatment of FLT3-ITD AML, and selective small molecule inhibitors, such as quizartinib, have been identified and trialled. However, clinical outcomes have been disappointing so far due to poor remission rates, also because of acquired resistance. A strategy to overcome resistance is to combine FLT3 inhibitors with other targeted therapies. In this study, we investigated the preclinical efficacy of the combination of quizartinib with the pan PI3K inhibitor BAY-806946 in FLT3-ITD cell lines and primary cells from AML patients. We show here that BAY-806946 enhanced quizartinib cytotoxicity and, most importantly, that this combination increases the ability of quizartinib to kill $CD34^{+}$ $CD38^{-}$leukemia stem cells, whilst sparing normal hematopoietic stem cells. Because constitutively active FLT3 receptor tyrosine kinase is known to boost aberrant PI3K signaling, the increased sensitivity of primary cells to the above combination can be the mechanistic results of the disruption of signaling by vertical inhibition.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal malignancy characterized by accumulation of immature blasts in the bone

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marrow and peripheral blood (Saultz and Garzon, 2016). Despite its high frequency in adults, mostly in the elderly, current treatment regimen still relies heavily on conventional chemotherapy, underscoring the unmet need for novel targeted therapies. Persistence of leukemia stem cells (LSC) drive AML leukemogenesis, responsible for drug resistance and disease relapse following conventional chemotherapy (Chopra and Bohlander, 2019; Welch et al., 2012). Advances over the past decade drastically improved our understanding of the disease biology and identified epigenomic dysregulation (Massett et al., 2021; Zampini et al., 2018) as well as chromosomal translocations and gene mutations such as Fms-like tyrosine kinase 3 (*FLT3*), DNA methyltransferase 3 A (*DNMT3A*), isocitrate dehydrogenase 1/1 (*IDH1/2*), Nucleophosmin 1 (*NPM1*) (Forghieri et al., 2020), Tet Methylcytosine Dioxygenase 1 (*TET1*), runt-related transcription factor 1 (*RUNX1*), and fusion genes (Port et al., 2014; Makkar et al., 2023) as common pathological features in human hematological malignancies, and therefore may represent putative druggable targets for the treatment of AML. Some small molecule inhibitors are indeed under clinical investigation and show promising efficacy.

Internal tandem duplication (ITD) mutations in FLT3 are one of the most common mutations in AML (Nakao et al., 1996) and are associated with poor prognosis (Thiede et al., 2002; Patnaik, 2018; Lagunas-Rangel, 2023), due to constitutive activation of FLT3 kinase and downstream pathways (Bogush et al., 2022; De Stefano et al., 2023), which includes the master regulator of survival, proliferation, and cell growth PI3K (Toker and Marmiroli, 2014; Mediani et al., 2016; Ruzzene et al., 2017; Bertacchini et., 2019; Braglia et al., 2020; Darici et al., 2020). Extensive commitments have led to the development of selective inhibitors against FLT3 (Daver et al., 2019; Scholl et al., 2020; Daver et al., 2021; Huang et al., 2021). Although these inhibitors initially demonstrated promising single-agent activity for the treatment of FLT3-ITD AML, with an overall survival longer than conventional chemotherapy, the beneficial response was short-lived mostly due to acquired resistance mechanisms (Cortes et al., 2019). A key strategy to overcome therapy resistance in cancer is combination therapy (Pisa and Kapoor, 2020; Vasan et al., 2019; Braglia et al., 2020). In particular, ongoing research indicates that combining two or more agents that target crucial survival pathways in AML (Fleischmann et al., 2021) may improve response rates and enable more durable remissions also in FLT3-ITD AML patients (Daver et al., 2019; Kennedy and Smith, 2020).

An accruing body of scientific knowledge has recognized deregulated PI3K/AKT/mTOR signaling as a central target in most cancer models (Braglia et al., 2020; Hillmann and Fabbro, 2019), as well as in AML (Darici et al., 2020; Evangelisti et al., 2020). This strategy has proven disappointingly difficult, though, and hitherto PI3K/AKT/mTOR inhibitors have not translated into clinical practice as monotherapy for AML patients. Still, the PI3K pathway remains a crucial target for FLT3-ITD AML, all the more so as this pathway is strongly associated with LSC survival and crosstalk between LSC and stromal cells associated bone marrow (BM) microenvironment which, in turn, plays a critical role in leukemogenesis providing protection of FLT3-ITD AML cells against FLT3 inhibitors, drug resistance and relapse (Zeng et al., 2012; Bolandi et al., 2021; Bruno et al., 2021; Ghiaur and Levis, 2017). Thus, co-targeting PI3K may sensitize FLT3-ITD AML cells to receptor tyrosine kinase inhibitor (RTKi)-lead targeted therapy. Consequently, in this study we assessed the efficacy of PI3K inhibitor BAY-806946 in combination with FLT3 inhibitor quizartinib in FLT3-ITD AML cell lines and primary patient cells.

2. Material and methods

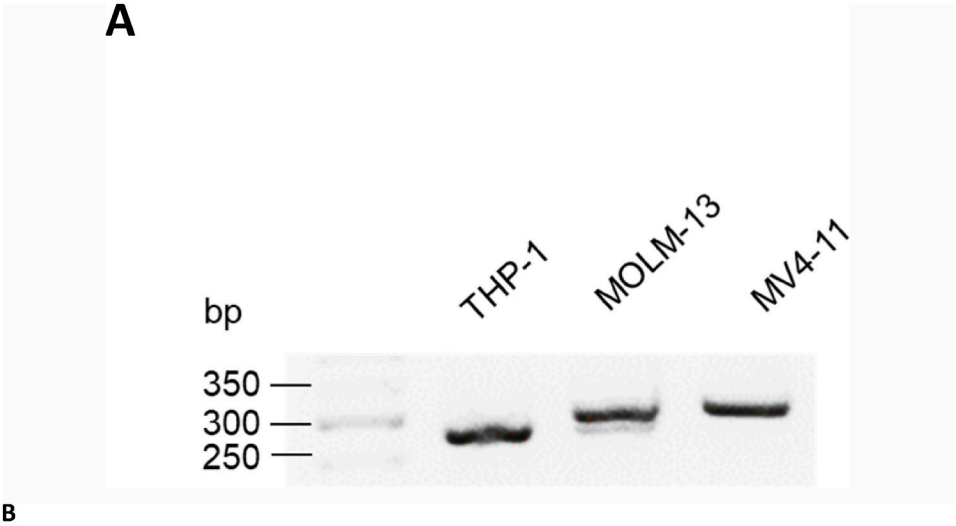
2.1. Cells culture and reagents

Quizartinib and BAY-806946 were purchased from Selleckchem and reconstituted at 10 mM in DMSO and 1 mM in 10% trifluoroacetic acid (TFA) (v/v), respectively. Drugs were stored at −20 °C. Cell lines THP-1, MOLM-13 and MV4-11 were purchased from DSMZ (Germany) and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (v/v) and 2 mM L-glutamine and stored at 37 °C, 5% CO_2 in a humidified incubator (Serafin et al., 2017). Cell line MS-5 was cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) (v/v) and 2 mM L-glutamine. All cell line culture reagents were purchased from Thermo Fisher Scientific. Primary cells were collected at diagnosis from AML patients following informed consent. All experimental procedures were performed in compliance with the guidelines of the European (86/609/EEC) and the Italian laws (D.L.116/92), and approved by the local ethic committee with protocols 4331/CE/AML study. Routine testing for all AML patients is performed at diagnosis for prognostic purposes and to guide the therapeutic choice. PBMC were purified from a bag of whole blood obtained from the Transfusional Center of the Policlinico, Modena, using Ficoll-Paque® Plus. The non-AML sample was obtained with written informed consent, in accordance with the declaration of Helsinki and with Greater Glasgow and Clyde NHS Trust Ethics Committee approval. Primary cells were cultured in StemSpan™ serum-free expansion medium (SFEM) (STEMCELL Technologies) and where indicated, supplemented with recombinant human growth factors IL-3, G-CSF and TPO (PeproTech).

2.2. Co-culture condition and cell growth analysis

In co-culture experiments, MS-5 cells were seeded at appropriate cell density to reach 70% confluency the next day. Following overnight incubation, culture medium was discarded and replaced by primary cell suspension. Cells were co-cultured with MS-5 overnight prior to any drug treatment.

Cell growth was assessed by colorimetric resazurin assay as described previously (Uzarski et al., 2017). Cell cycle analysis and assessment of apoptosis in cell lines was performed as described in our previous report (Darici et al., 2021).



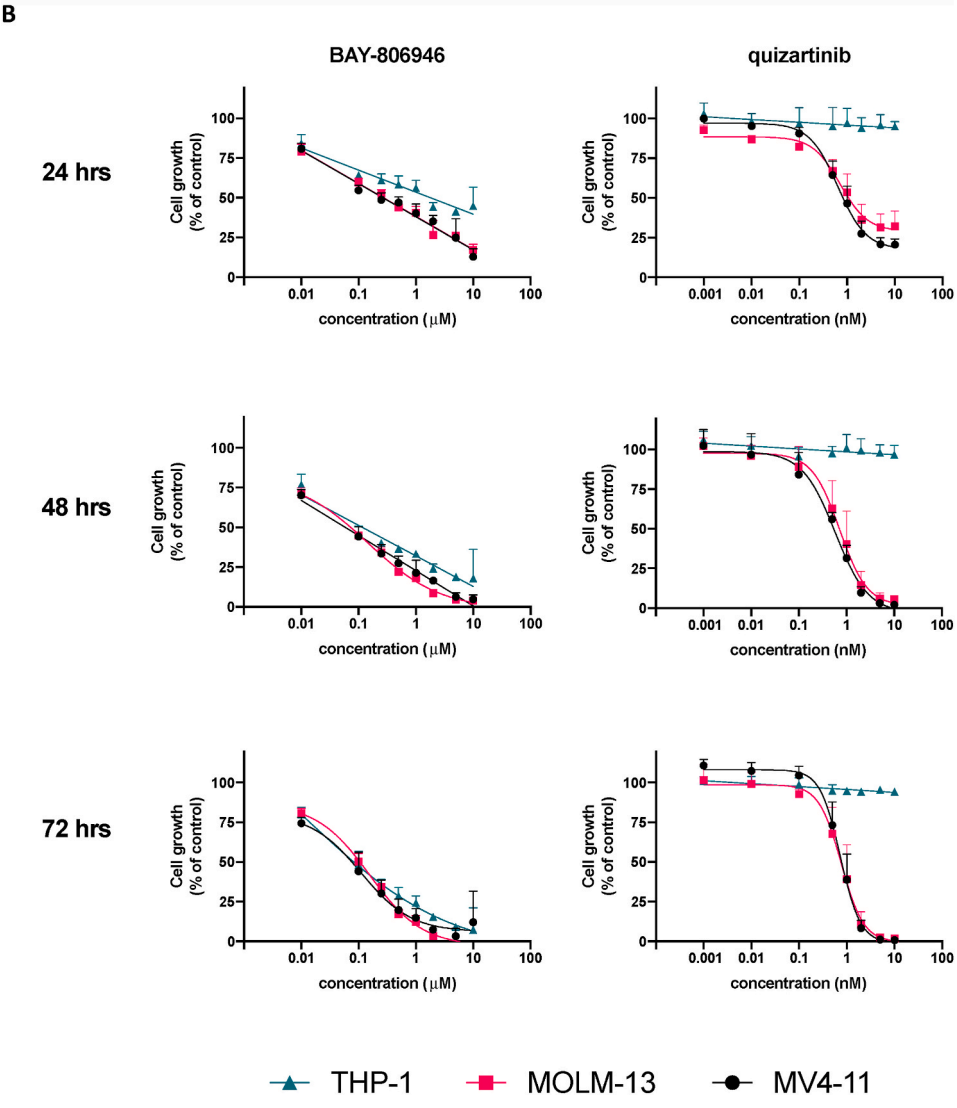


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Fig. 1. BAY-806946 or quizartinib induce growth inhibition in human FLT3-ITD AML cell line.
a) The PCR product from the human FLT3-ITD AML cell lines MOLM-13 and MV4-11 confirm the expected 21 base pairs (bp) and 30 bp insertion, respectively. As MOLM-13 cells express both the mutated and the wild type allele, a second band is detectable. THP-1 was included as FLT3 wild type control. B) THP-1, MOLM-13 and MV4-11 cells were exposed to increasing concentrations of BAY-806946 or quizartinib, as indicated. Growth inhibition was assessed at 24, 48 or 72 h by a resazurin-based metabolic assay. Cell growth curves represent the averages of three independent replicates. Error bars represent average ± SD for each drug concentration.

2.3. Flow cytometric analysis of primary cells

Non-adherent cells were collected by pipetting and adherent cells by trypsinization. Cells were washed with PBS and resuspended in 100 μL antibody staining mix (CD45, CD33, CD34, CD38, annexin V and DAPI) for 10 min on ice in the dark before being analyzed on the FACSCantoII flow cytometer (BD Biosciences). All antibodies were purchased from BD Biosciences). Prior to analysis, 50 μL of CountBright™ Absolute Counting Beads (Thermo Fisher Scientific) were added to each tube to assess cell count. Data was acquired using BD FACS Diva Software and quantification was carried out using FlowJo software.

2.4. Luminex analysis

Cytokine profiling was performed using the Cytokine 25-Plex Human ProcartaPlex™ Panel 1 B (Thermo Fisher Scientific) according to manufacturer's instruction. The MAGPIX® System (Luminex® xMAP® Technology) was used to read the plate. ProcartaPlex™ Analyst software was used for data analysis.

2.5. RT-qPCR analysis

For PCR amplification of FLT3-ITD DNA, a mastermix was prepared using the GoTaq® G2 DNA Polymerase kit (Promega), dNTPs and target-specific primers (Forward primer sequence 5′ to 3′ GGTGACCGGCTCCTCAGATA; Reverse primer sequence 5′ to 3' CGGCAACCTGGATTGAGACT). 1 μL gDNA was added to the mastermix per reaction at the following thermal cycling conditions.

Step	Temperature (°C)	Duration (mins)	Cycles
1	95	2:00	1
2	95	0:30	30
3	62	0:30	
4	72	0:30	
5	72	2:00	1

The PCR product was run on a 3% (w/v) agarose gel made with 1x TAE buffer plus 1:10,000 SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific) for 60 min at 100 V. In a separate lane, 6 μL DNA ladder was loaded. The gel was imaged using the Odyssey Fc Imaging System.

2.6. Statistical analysis

The data are shown either as mean values or as individual values. Error bars represent the standard deviation (SD). All statistical analysis was performed using the GraphPad Prism software. To compare multiple groups, paired one-way ANOVA with multiple comparisons test was performed. A p-value of <0.05 was considered statistically significant.

Table 1
Summary of IC50 values of FLT3 wildtype (THP-1) and FLT3-ITD (MOLM-13 and MV4-11) AML cell lines treated with BAY-806946 or quizartinib. Stated IC50 values (nM) represent average of three independent replicates.

Cell line	Compound	24 h	48 h	72 h
THP-1	BAY-806946	1801.3	117.5	17.98
	quizartinib	not reached[a]	not reached[a]	not reached[a]
MOLM-13	BAY-806946	273.2	145.9	155.9
	quizartinib	0.7	0.7	0.8
MV4-11	BAY-806946	267.3	59.2	110.9
	quizartinib	0.6	0.6	0.7

[a] 'not reached' > 10,000 nM quizartinib.

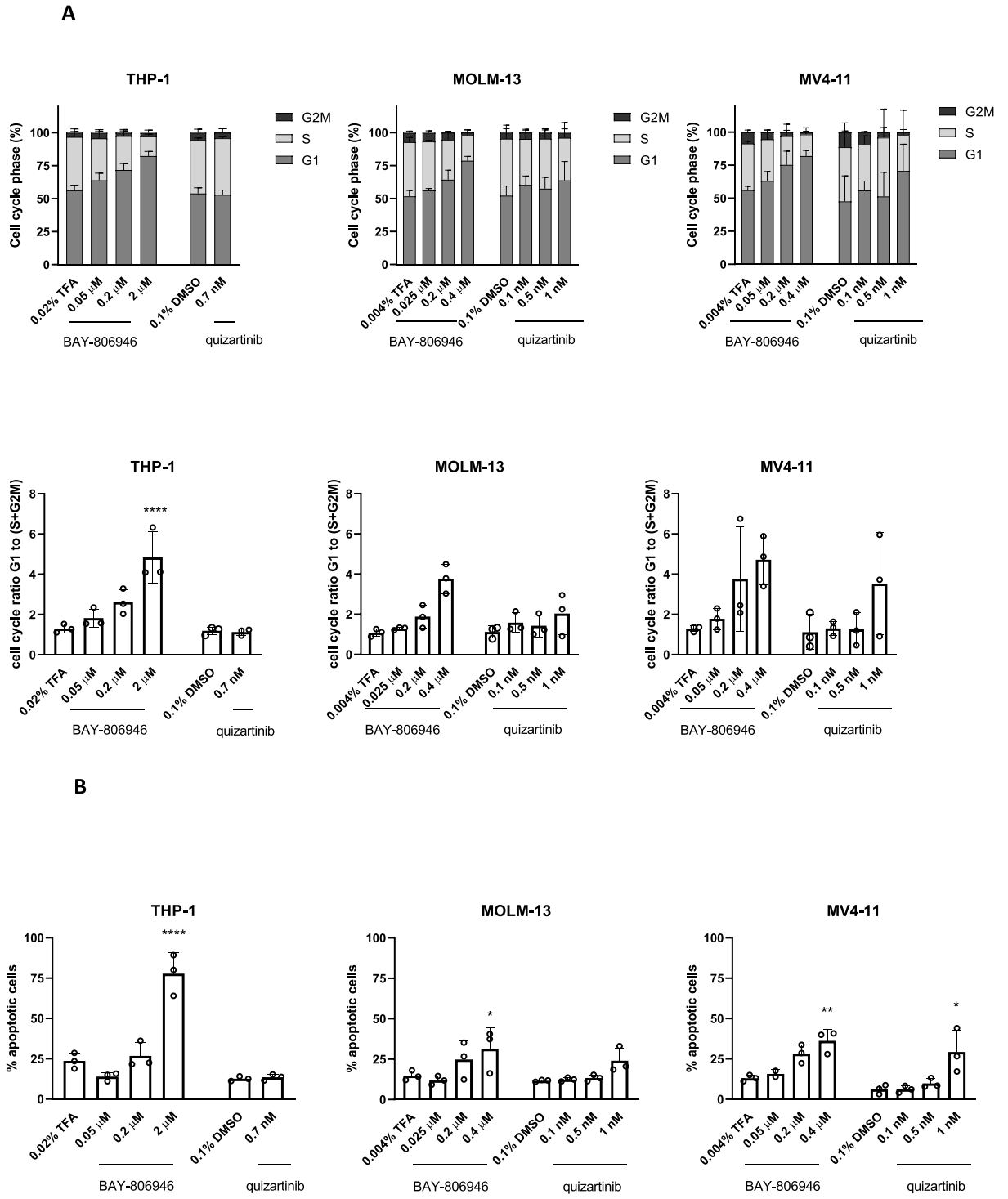

Fig. 2. Effect of BAY-806946 and quizartinib on cell cycle and apoptosis.
FLT3 wildtype (THP-1) and FLT3-ITD (MOLM-13 and MV4-11) cell lines were exposed to BAY-806946 or quizartinib, as indicated, for 48 h. THP-1 cells were treated with quizartinib to show there is no detectable off-target effect. A) Cell cycle was measured by propidium iodide staining and flow cytometry. Bars represent the average fraction ± SD in each cell cycle phase (upper panel) and bars in graphs represent the ratio of G1 to (S + G2M) of three independent replicates (lower panel). Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparisons test (****$p \leq 0.0001$). B) Apoptosis was measured by annexinV/DAPI staining and flow cytometry. Each bar represents the average ± SD percentages of apoptotic cells of three independent replicates. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparisons (*$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$).

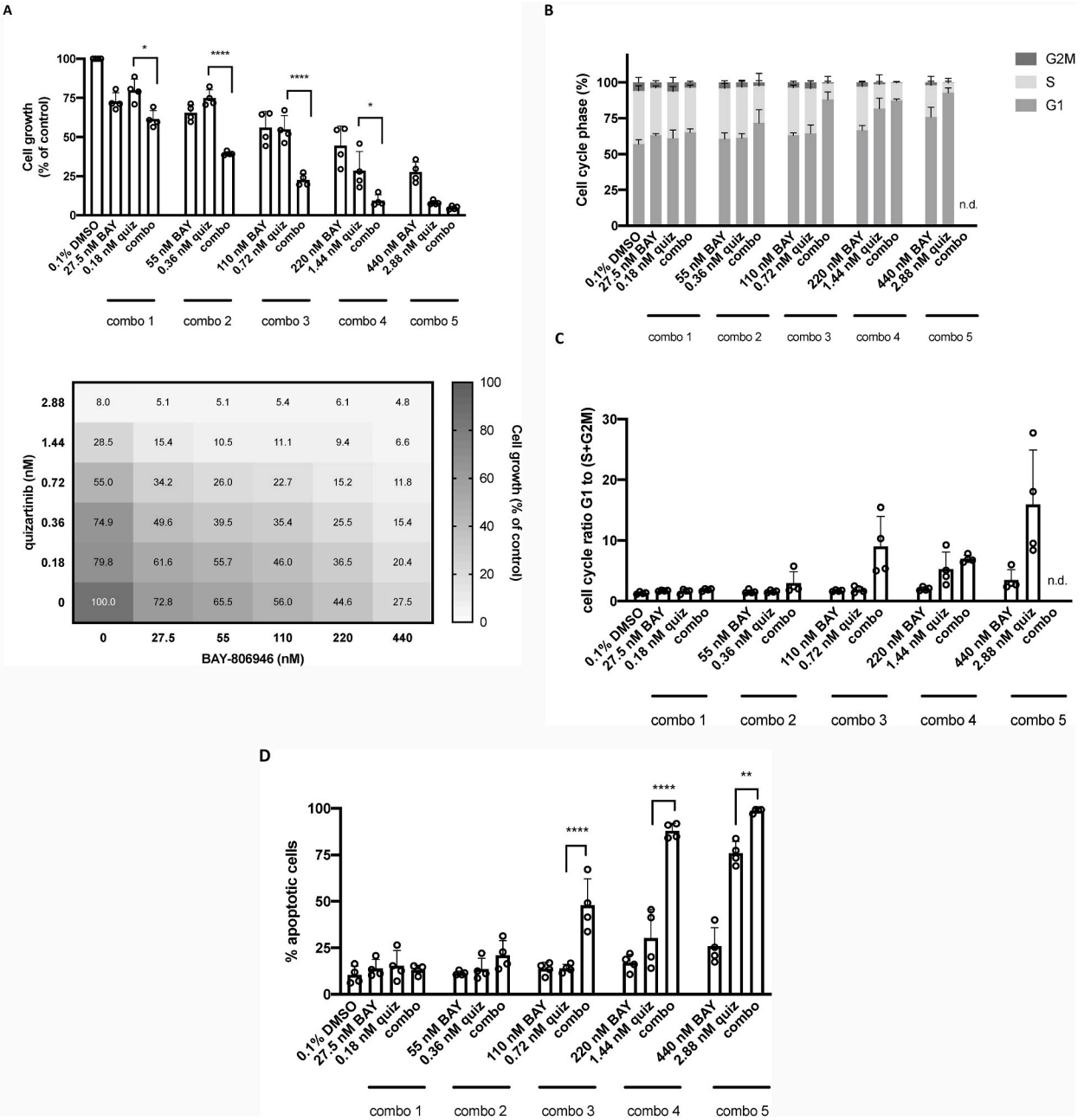

Fig. 3. Combination of quizartinib with BAY-806946 inhibits cell growth through enhanced G1 cell cycle arrest and apoptosis in FLT3-ITD AML cell lines.

FLT3-ITD cells were exposed to increasing concentrations of quizartinib (quiz) and BAY-806946 (BAY) around their respective drug IC50 concentrations for 48 h, either alone or in combination. 1x IC50 were 110 nM (BAY) and 0.72 nM (quiz). A) Growth inhibition was measured by resazurin-based cell metabolism assay. Bar graphs (upper part) and heatmap (lower part) represent the average percentage of inhibition of cell growth (normalized to vehicle control) of four independent replicates, ±SD for each drug concentration. B) Stacked bar graphs representing the average fraction in each cell cycle phase, ±SD, after propidium iodide staining and flow cytometric analysis. The G1 to (S + G2M) ratio of four independent replicates, ±SD, is shown in panel C. The highest drug concentration combination was toxic and therefore not included (n.d.). D) Apoptosis was measured by annexinV/DAPI staining followed by flow cytometry. Each bar represents the average percentages of apoptotic cells ± SD of four independent replicates. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. (**$p \leq 0.01$, ****$p \leq 0.0001$).

3. Results and discussion

3.1. Combination of quizartinib with BAY-806946 exerts enhanced cytotoxicity in FLT3-ITD AML cell lines

In our previous report, we have demonstrated that parallel blockade of constitutively active Flt3 and PI3K/mTOR signaling displayed synergistic cytotoxicity in vitro in FLT3-ITD AML cell lines (Darici et al., 2021). Due to the importance of this signaling axis in cancer and to the availability of several specific compounds, here we aimed to explore the potential of pan-PI3K inhibitor BAY-806946 to enhance the efficacy of quizartinib in FLT3-ITD AML primary cells and in human AML-derived cell lines MOLM-13 and MV4-11 expressing FLT3-ITD (Quentmeier et al., 2003), as confirmed by PCR (Fig. 1a).

First, we evaluated the selectivity of the inhibitor, by comparing the efficacy of BAY-806946 and quizartinib on the above cell lines versus FLT3 wildtype THP-1. As expected in accordance to previous reports (Aikawa et al., 2020; Darici et al., 2021), quizartinib selectively induces antileukemic effects in FLT3-ITD AML cells without affecting FLT3 wildtype cells (IC50 0.7 nM and 0.6 nM in MOLM-13 and MV4-11, respectively) (Fig. 1b). Conversely, BAY-806946 displayed a clear concentration-and time-dependent decrease of cell growth in both FLT3-ITD and FLT3 wt AML cell lines, with varying efficacy, detected by a resazurin-based cell viability assay (IC50 117.5, 145.9, 59.2 in THP-1, MOLM-13, MV4-11, respectively) (Fig. 1b and Table 1).

Next, we investigated the effect of these drugs on cell cycle progression by FACS analysis. It is clear that growth inhibition by quizartinib was mainly through G1 arrest (Fig. 2A–B). On the other hand, BAY-806946 elicited not only G1 arrest but also apoptosis (arrest (Fig. 2A–B, Figs. 1S and 2S).

To explore whether BAY-806946 potentiates the efficacy of quizartinib, cells were exposed to a range of doses around their respective IC50 for 48 h. Concomitant treatment drastically inhibited cell growth mainly through apoptosis, at concentrations in the nanomolar range (Fig. 3). In particular, 0.72 nM quizartinib with 110 nM BAY-806946 induced 77.3% inhibition of cell growth, as detected by resazurin-based assay (Fig. 3A), cell cycle arrest in G1 phase evaluated by flow cytometry (Fig. 3B–C), and 47.9% apoptosis (Fig. 3D), assessed by flow cytometry.

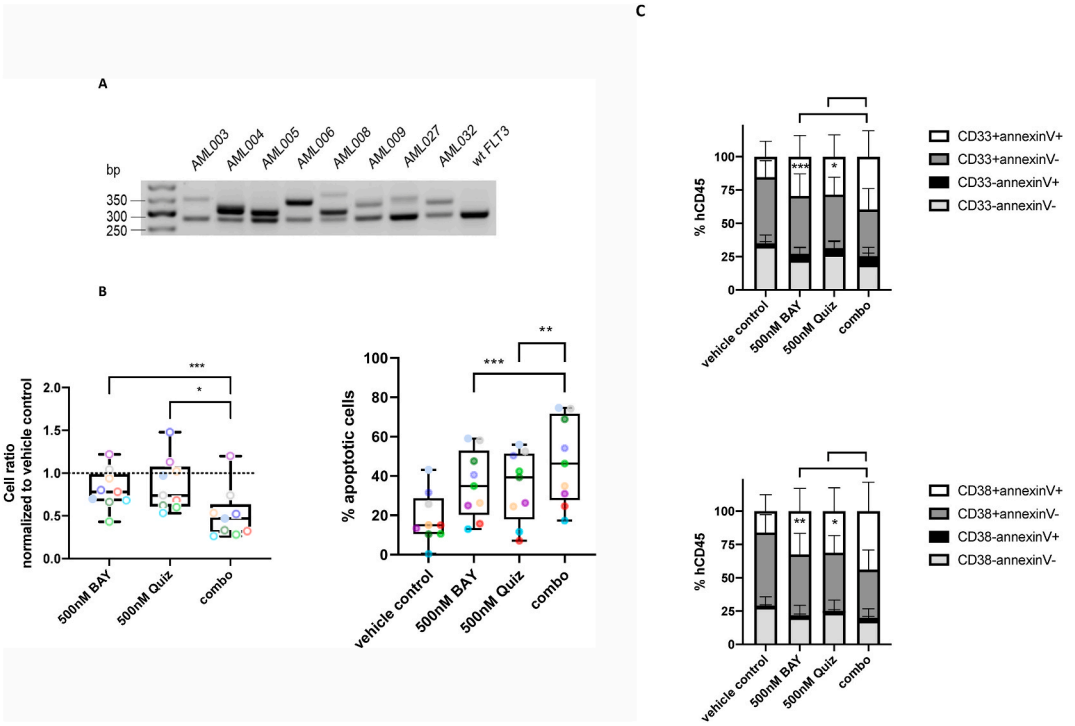

Fig. 4. Combination treatment induces growth inhibition and apoptosis in FLT3-ITD AML primary patient cells co-cultured on stroma.
Primary cells from 9 individual FLT3-ITD AML patients were cultured overnight in the presence of stroma in SFEM, then treated with quizartinib (Quiz) and BAY-806946 (BAY) alone or in combination at the indicated concentration for 48 h. A) FLT3-ITD mutational status of primary patient samples was confirmed by PCR. THP-1 cell line was included as FLT3 wildtype control. B) Box plots show $CD45^+$ cell count by flow cytometry, normalized to vehicle control, analyzed for cell growth (left) or apoptosis (by annexinV/DAPI, right). C) Cells were stained with surface markers CD45, CD33, CD38 and viability markers annexinV/DAPI, followed by flow cytometry. Stacked bar graphs represent the percentage of CD33/annexinV (upper panel) or CD38/annexinV (lower panel) expression in the $CD45^+$ subpopulation. Statistical significance was calculated by one-way ANOVA (using Geisser-Greenhouse correction) followed by Tukey's multiple comparisons test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

3.2. Combination treatment induces enhanced growth inhibition and cytotoxicity in FLT3-ITD AML primary patient cells ex vivo

Having shown that BAY-806946 potentiates the efficacy of quizartinib in FLT3-ITD AML cell lines, we evaluated the same combination in primary cells from FLT3-ITD AML (Fig. 4A) cultured on stroma (Griessinger et al., 2014), a setting more relevant to the clinic. Cells were treated concomitantly with quizartinib and BAY-806946 for 48 h, and cell growth and apoptosis were measured using flow cytometry as above. In good agreement with the results on the cell lines, combination treatment displayed enhanced growth inhibition caused by apoptosis, compared to either monotherapies (Fig. 4B). It is worth noting that the drug response amongst patient samples is quite variable, though, reflecting the heterogeneity of AML. To confirm that the cells undergoing apoptosis were in fact leukemic cells, we stained them with myeloid markers CD33 and CD38. Leukemic cells were indeed targeted, as combination treatment significantly increased the CD33+annexinV+ ($p = 0.0003$) and CD38+annexinV+ ($p = 0.0013$) subsets respective to BAY-806946 treatment alone (Fig. 4C).

3.3. Leukemia stem cells are targeted by combined Flt3 and PI3K inhibition sparing normal cells

Then, to assess whether combination treatment eradicates also LSC, which are at the apex generating disease progeny and therapy

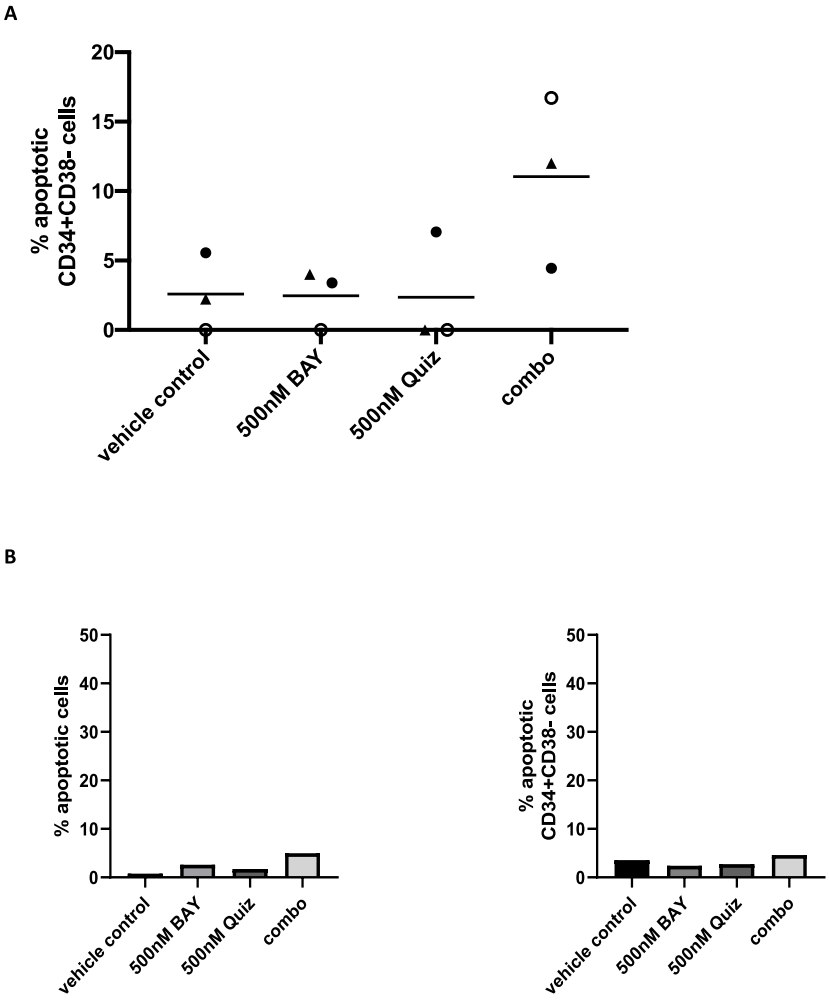

Fig. 5. Combination treatment induces apoptotic effects in the stem cell-like $CD34^+CD38^-$subpopulation of FLT3-ITD AML primary cells co-cultured on stroma with exogenous physiological growth factors, but spares normal peripheral blood mononuclear cells.
Cells were cultured overnight in the presence of stroma in SFEM supplemented with IL-3, G-CSF and TPO at 1 ng/mL, then treated with quizartinib (Quiz) and BAY-806946 (BAY) for 48 h as described above. Apoptosis was assessed by annexinV/DAPI followed by flow cytometry. A) Apoptosis evaluation in $CD45^+$ stem cell-like $CD34^+CD38^-$cells from 9 FLT3-ITD AML primary cell. Box plots depict the mean percentage of apoptosis in the 3 out of 9 samples with measurable $CD34^+CD38^-$cells. Statistical significance was calculated by one-way ANOVA (using Geisser-Greenhouse correction) followed by Tukey's multiple comparisons test. B) Apoptosis in peripheral blood mononuclear cells (PBMC) from healthy donors (left) and in primary $CD34^+CD38^-$cells representing normal HSC non-AML cells (right).

resistance, physiological concentrations of growth factors (IL-3, G-CSF and TPO) were added to primary cells co-cultured on stroma to support their maintenance during drug treatment (Griessinger et al., 2016). Cells were stained with LSC markers CD34 and CD38, and monitored by flow cytometry (Bonnet and Dick, 1997; Lapidot et al., 1994; Bonnet, 2017). Although a CD34+/38- population was detected only in 3 out of 9 samples, it is interesting to notice that combination treatment strongly enhanced apoptosis in 2 out 3 samples (Fig. 5A).

We finally checked the cytotoxicity of the two inhibitors to normal cells. To this end, peripheral blood mononuclear cell (PBMC) from healthy donors were co-cultured on stroma and treated with quizartinib (500 nM) and BAY-806946 (500 nM) as above for 48 h (Fig. 5B, left panel). Cytotoxicity assessment by flow cytometry revealed only a slight decrease of cell viability in healthy PBMC. More importantly, the $CD34^{+}CD38^{-}$subset of non-AML patient cells, representing normal HSC, were unaffected (Fig. 5B, right panel).

3.4. Combination treatment and/or addition of exogenous physiological growth factors alters the cytokine release profile of FLT3-ITD AML primary patient cells

Finally, we considered that molecules secreted by AML cells are deemed to play a role in autocrine and paracrine regulation of AML growth and survival (Behrmann et al., 2018; Oliveira et al., 2019). Because co-culture with stromal cells and addition of exogenous PGF was shown to attenuate response to combination treatment in FLT3-ITD AML primary patient cells, it was investigated whether the media conditioned by AML cells is altered in cytokine secretion profile. Cells from nine primary patients co-cultured with MS-5 cells plus/minus PGF and treated for 48 h with BAY-806946 and quizartinib alone or in combination. In total, the concentration of 25 different cytokines was measured and the results are summarized in the heatmap (Fig. 6) and in Tables 2 and 3. Granulocyte-macrophage colony stimulating factor (GM-CSF) (2/9), IL-1β (7/9), IL-1RA (8/9), and IL-6 (2/9) were detectable in the culture media using Luminex platform, while other cytokines were below detection limit or <1 pg/mL. Variability of cytokine levels amongst patient samples reflects the heterogeneity of AML. In particular, our results show that IL-RA content is higher in co-cultured AML cells plus PGF (vehicle control: 132.8 pg/mL) respective to SFEM (vehicle control: 114.9 pg/mL). Furthermore, IL-1RA level following combination treatment was increased respective to vehicle control (in SFEM: 133.1 pg/mL; +PGF: 142.1 pg/mL). Above all, sample AML005 showed striking levels of IL-1RA compared to the other evaluated patient samples. With regards to IL-1β, secretion levels were reduced in the presence of PGF (vehicle control: 0.7 pg/mL) compared to SFEM (vehicle control: 2 pg/mL), and following

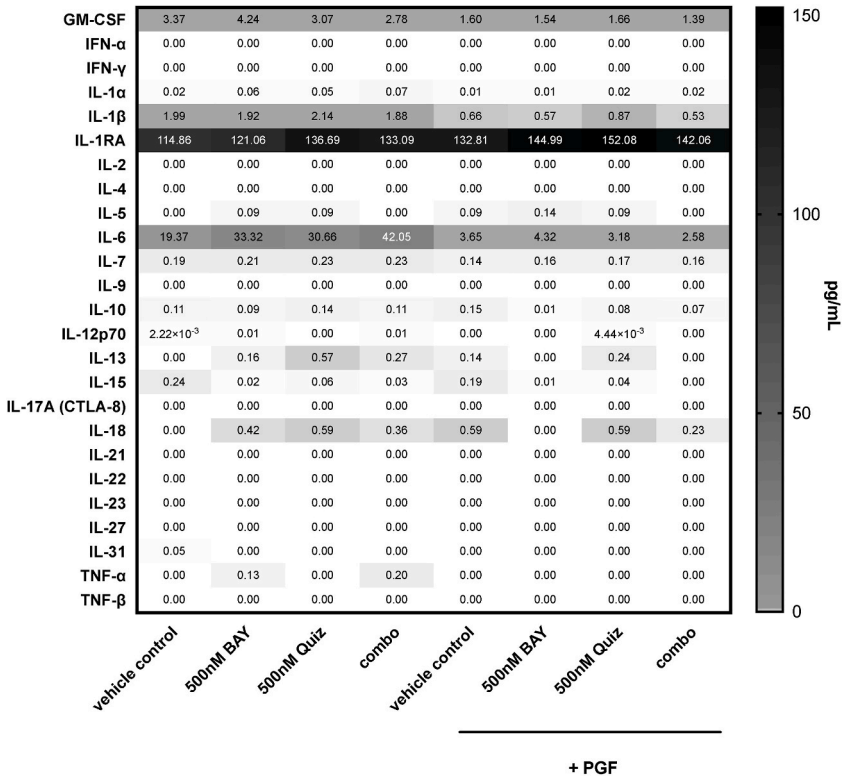

Fig. 6. Cytokine secretion levels in media harvested from FLT3-ITD AML primary patient cells.
FLT3-ITD AML cells were cultured in the presence of stroma in SFEM supplemented with or without IL-3, G-CSF and TPO at 1 ng/mL, as indicated. Following overnight co-culture, cells were treated with quizartinib (Quiz) and BAY-806946 (BAY) alone or in combination at the indicated concentration in technical duplicates for 48 h. Supernatants were collected for detection and quantification of the indicated cytokines. Heat map represents the mean concentration (pg/mL) of the indicated cytokines measured in nine individual FLT3-ITD AML primary patients.

Table 2
Summary of cytokine secretion levels in primary cells co-cultured with stromal cells.
The cytokine secretion levels for 9 FLT3-ITD AML primary patient cells co-cultured with stromal cells in SFEM following combination treatment is summarized in the table.

cytokine	#N	vehicle control			BAY-806946			Quizartinib			Combination			*p*-value
		Range (pg/mL)	Mean conc. (pg/mL)	SD	Range (pg/mL)	Mean conc. (pg/mL)	SD	Range (pg/mL)	Mean conc. (pg/mL)	SD	Range (pg/mL)	Mean conc. (pg/mL)	SD	
GM-CSF	9	0–19.2	3.4	7.0	0–27.4	4.2	9.4	0–15.0	3.1	6.1	0–13.3	2.8	5.5	n.s.
IFN-α	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IFN-γ	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-1α	9	0–0.1	0.0	0.0	0–0.2	0.1	0.1	0–0.2	0.1	0.1	0–0.2	0.1	0.1	n.s.
IL-1β	9	0–7.2	2.0	2.4	0–7.0	1.9	2.4	0–9.1	2.1	2.9	0–8.7	1.9	2.7	n.s.
IL-1RA	9	0–464.3	114.9	151.7	0–452.3	121.1	151.6	0–514.0	136.7	171.0	0–510.4	133.1	170.9	n.s.
IL-2	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-4	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-5	9	0	0.0	0.0	0–0.1	0.1	0.3	0–0.9	0.1	0.3	0	0.0	0.0	n.s.
IL-6	9	0–131.4	19.4	44.3	0–218.2	33.3	72.2	0–214.9	30.7	70.7	0–294.7	42.0	97.0	n.s.
IL-7	9	0–0.5	0.2	0.2	0–0.5	0.2	0.2	0–0.6	0.2	0.2	0–0.5	0.2	0.2	n.s.
IL-9	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-10	9	0–0.8	0.1	0.3	0–0.7	0.1	0.2	0–0.7	0.1	0.3	0–0.7	0.1	0.2	n.s.
IL-12p70	9	0	0.0	0.0	0–0.1	0.0	0.0	0	0.0	0.0	0–0.1	0.0	0.0	n.s.
IL-13	9	0	0.0	0.0	0–1.4	0.2	0.5	0–5.1	0.6	1.7	0–2.4	0.3	0.8	n.s.
IL-15	9	0	0.2	0.2	0–0.1	0.0	0.0	0–0.3	0.1	0.1	0–0.2	0.0	0.1	n.s.
IL-17 A (CTLA-8)	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-18	9	0	0.0	0.0	0–3.8	0.4	1.3	0–5.3	0.6	1.8	0–3.2	0.4	1.1	n.s.
IL-21	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-22	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-23	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-27	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-31	9	0–0.4	0.0	0.1	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
TNF-α	9	0	0.0	0.0	0–1.2	0.1	0.4	0	0.0	0.0	0–1.8	0.2	0.6	n.s.
TNF-β	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.

LOD; limit of detection; n.s.; not significant; PGF; physiological growth factors; p-values (two-way ANOVA) for monotherapy with quizartinib versus combination therapy are represented in the right column.

Table 3
Effect of exogenous PGF on cytokine secretion levels.
The table summarize the cytokine secretion levels for 9 FLT3-ITD AML primary patient cells co-cultured with stromal cells in the presence of exogenous physiological growth factors (PGF) following combination treatment.

cytokine	#N	vehicle control			BAY-806946			Quizartinib			Combination			*p*-value
		Range (pg/mL)	Mean conc. (pg/mL)	SD	Range (pg/mL)	Mean conc. (pg/mL)	SD	Range (pg/mL)	Mean conc. (pg/mL)	SD	Range (pg/mL)	Mean conc. (pg/mL)	SD	
GM-CSF	9	0–7.5	1.6	3.2	0–7.9	1.5	3.1	0–8.4	1.7	3.3	0–6.5	1.4	2.8	n.s.
IFN-α	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IFN-γ	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-1α	9	0	0.0	0.0	0–0.1	0.0	0.0	0–0.1	0.0	0.0	0–0.1	0.0	0.0	n.s.
IL-1β	9	0–1.9	0.7	0.7	0–2.9	0.6	0.9	0–2.9	0.9	1.0	0–2.6	0.5	0.9	n.s.
IL-1RA	9	0–533.2	132.8	176.0	0–505.8	145.0	173.0	0–553.1	152.1	189.4	0–505.3	142.1	174.6	n.s.
IL-2	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-4	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-5	9	0–0.9	0.1	0.3	0–1.3	0.1	0.4	0–0.9	0.1	0.3	0	0.0	0.0	n.s.
IL-6	9	0–18.0	3.7	6.7	0–26.9	4.3	9.3	0–16.0	3.2	6.4	0–18.4	2.6	6.1	n.s.
IL-7	9	0–0.3	0.1	0.1	0–0.4	0.2	0.2	0–0.4	0.2	0.2	0–0.4	0.2	0.2	n.s.
IL-9	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-10	9	0–0.9	0.2	0.3	0–0.1	0.0	0.0	0–0.7	0.1	0.2	0–0.7	0.1	0.2	n.s.
IL-12p70	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-13	9	0–1.2	0.1	0.4	0	0.0	0.0	0–2.2	0.2	0.7	0	0.0	0.0	n.s.
IL-15	9	0–0.5	0.2	0.2	0–0.1	0.0	0.0	0–0.2	0.0	0.1	0	0.0	0.0	n.s.
IL-17 A (CTLA-8)	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-18	9	0–5.3	0.6	1.8	0	0.0	0.0	0–5.3	0.6	1.8	0–2.1	0.2	0.7	n.s.
IL-21	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-22	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-23	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-27	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
IL-31	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
TNF-α	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.
TNF-β	9	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	n.s.

"0" indicates below limit of detection; n.s.; not significant; PGF; physiological growth factors; p-values (two-way ANOVA) for monotherapy with quizartinib versus combination therapy are represented in the right column.

combination treatment; these levels were further reduced (in SFEM: 1.9 pg/mL; +PGF: 0.5 pg/mL). A similar pattern was observed with GM-CSF and IL-6 where co-culture in the presence of PGF reduced cytokine levels respective to co-culture in SFEM. Interestingly, in SFEM, both BAY-806946 and quizartinib as monotherapy increased IL-6 levels respective to vehicle control (33.3 pg/mL (BAY), 30.7 pg/mL (quiz) versus 19.4 pg/mL (vehicle), which was further elevated with the drug combination (42 pg/mL). The opposite effect was observed in the presence of PGF where drug treatment decreased IL-6 levels, most strikingly with sample AML009. Notably, the level of GM-CSF levels was only measurable in two samples.

4. Conclusions

In our present study, we investigated whether co-targeting of the PI3K/AKT/mTOR pathway using BAY-806946 could potentiate the efficacy of selective FLT3 inhibitor quizartinib in vitro using FLT3-ITD AML cell lines and primary cells. FLT3 kinase is regarded an attractive druggable target for the treatment of FLT3-ITD AML and extensive commitments have identified selective and potent FLT3 inhibitors such as quizartinib that failed to induce durable remissions in clinical trials either alone or in combination with other therapeutics as a result of primary and secondary resistance mechanisms. Thus, improved combination strategies are urgently required. There is increasing appreciation that resistance to FLT3 inhibition is mediated by PI3K/AKT/mTOR and that blocking this pathway may sensitize FLT3-ITD AML cells to FLT3 inhibitors. As such, it has been reported that FLT3 inhibitor resistant FLT3-ITD AML cell lines display aberrant activation of PI3K/AKT/mTOR, and inhibition of this pathway extended survival of sorafenib-resistant mice in vivo (Lindblad et al., 2016). More recently, it has been uncovered that the BM microenvironment protects FLT3-ITD AML cells from apoptosis following FLT3 inhibition, mediated by the ataxia-telangiectasia mutated (ATM)/mTOR pathway (Park et al., 2022). Remarkably, co-targeting these mediators abrogated BM-mediated stromal protection and exert enhanced elimination of FLT3-ITD AML cells.

Consistent with these findings, our work showed that the drug combination acts synergistically for biological outcomes, displaying enhanced cytotoxicity in both FLT3-ITD AML cell lines and primary cells. More importantly, there are indications that the drug treatment may eradicate AML LSC whilst moderately affecting normal cells that require PI3K/AKT/mTOR activity for normal cellular function. As PI3K signaling reactivation has been recognized as a major drawback of PI3K signaling inhibitor-lead therapy (Braglia et al., 2020), deeper investigation is mandatory to determine whether BAY-806946 induces permanent blockade of PI3K and downstream pathway, thus avoiding paradoxical reactivation.

CRediT authorship contribution statement

Salihanur Darici: planned and carried out experimental work, cell culture and cell growth assay, flow cytometry for cell cycle and apoptosis analysis, and statistical, performed, Writing – original draft, Writing – review & editing, Conceptualization. **Heather G. Jørgensen:** Supervision. **Xu Huang:** conceptualized and designed experiments, Supervision, Funding acquisition. **Valentina Serafin:** contributed to, Methodology, Investigation, statistical analysis of supplementary figures results, Funding acquisition. **Ludovica Antolini:** Methodology, Investigation. **Patrizia Barozzi:** collected and characterized patient samples. **Mario Luppi:** collected and characterized patient samples. **Fabio Forghieri:** collected and characterized patient samples. **Sandra Marmiroli:** conceptualized and designed experiments, Supervision, performed, Writing – original draft, Writing – review & editing, Conceptualization, Funding acquisition. **Manuela Zavatti:** performed cell culture and treatments, prepared pellets and cell extracts.

Declaration of competing interest

None

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbior.2023.100974.

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