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Combined Effect of 2nd Generation Inhibitors on FLT3ITD Phosphorylation

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Abstract: AML (acute myeloid leukemia) is an aggressive hematopoietic malignancy with multiple signaling pathways contributing to its pathogenesis. A key role in of these pathways is the FLT3 (FMS-like tyrosine kinase receptor-3). Activation of the FLT3ITD (internal tandem duplication of FLT3) leads to decreased progression and low survivability rate. Targeting the kinase activity of FLT3 with inhibitory compounds can be used as an obvious therapeutic option. The second generation inhibitors have shown enhanced FLT3 specificity and good results in targeting AML. The aim of this study is to elucidate the combined effects of these inhibitors on FLT3ITD signal transduction.

Key words: Inhibitors, FLT3ITD, phosphorylation, AML.

1. Introduction

FLT3 (FMS-like tyrosine kinase-3) is a member of the type 3 RTK (receptor tyrosine kinase) family and is overly expressed in pluripotent hematopoietic stem cells and progenitors (Fig. 1). It is also found in high levels in the blast cells of patients with AML (acute myeloid leukemia). These poorly differentiated precursor cells cease to function normally and disrupt normal hematopoiesis causing infection, bleeding and multiple organ dysfunction.

The FMS-like tyrosine kinase-3 gene encodes a membrane-bound receptor kinase which plays a very important role for the normal hematopoiesis. FLT3 mutates in about 1/3 of acute myeloid leukemia patients, 5~10% in myelodysplasia and 1~3% of acute lymphoblastic leukemia, making FLT3 one of the most mutated genes in hematopoietic malignancies. Small internal tandem duplications in the JM (juxtamembrane) part of the FLT3 gene in AML patients have been reported by Nakao et al. [1]. It was reported that ITD (internal tandem duplication) led to the activation of the receptor through constitutive dimerization [2].

The activation of FLT3 is initiated by the binding of the ligand (FLT3 ligand FL) to the extracellular domain, which in turn promotes the dimerization and the juxtapositioning of the cytoplasmic domain. The transphosphorylation of tyrosine occurs which releases the autoinhibition mediated by the JM domain [3]. The kinase activity of FLT3 is regulated by tyrosine phosphatases that dephosphorylatestyrosines 589 and 591 in the JM domain. This gives way to the receptor to re-adopt its autoinhibitory conformation. FLT3 is also regulated negatively by its own rapid internalization and polyubiquitination, which leads to trafficking and proteasomal degradation [4].

2. Abnormal Signaling of FLT3 in Acute Myeloid Leukemia

Numerous studies have shown that a high percentage of AML patients carry FLT3-ITD sequence mutations (about 25% of patients) inserted in the JM domains [5]. These patients have a poorer prognosis compared to patients that express the WT (wild type) FLT3, even though this is normally dependent on the allelic ratio of FLT3. The outcome of these mutations is

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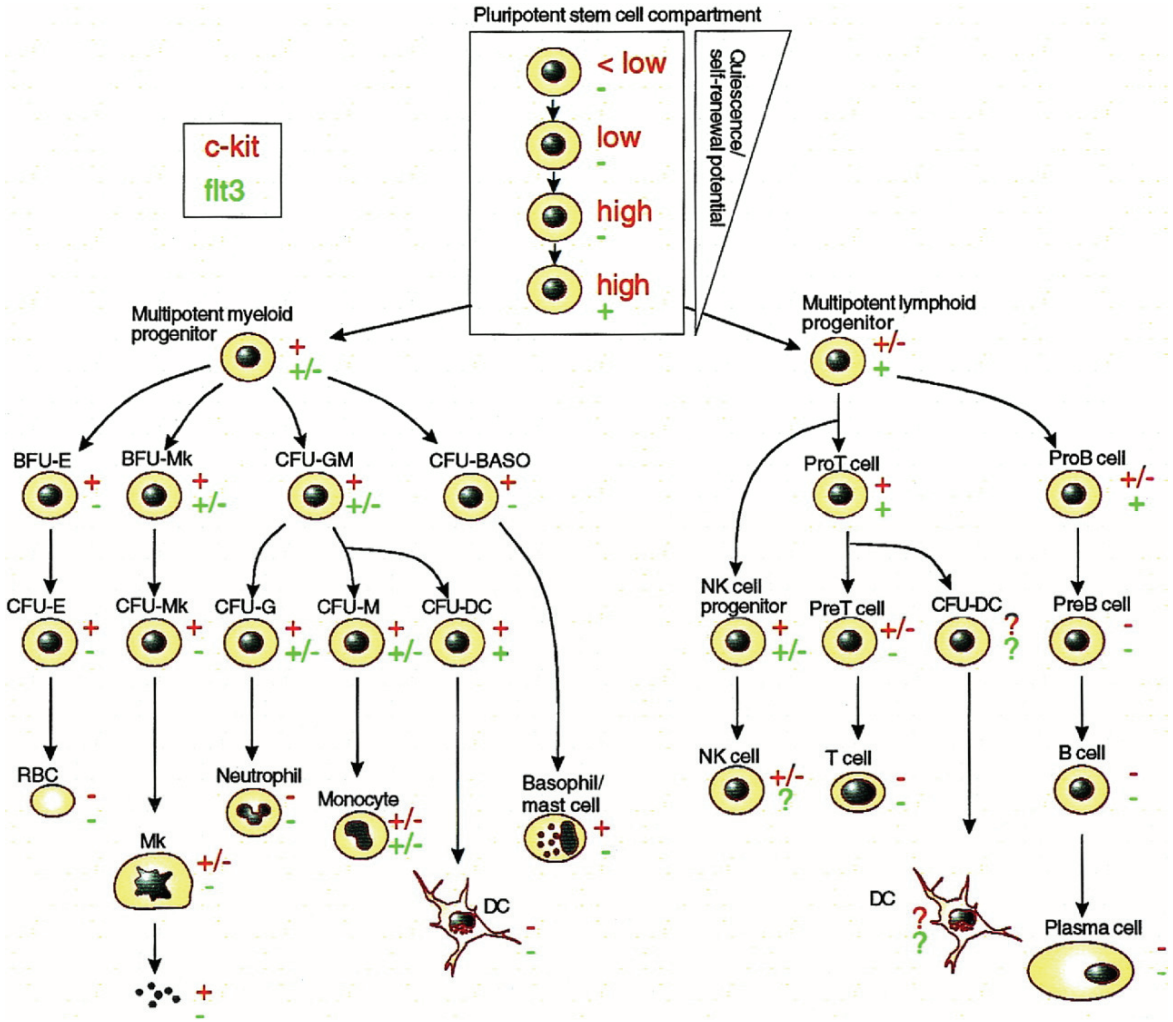

Fig. 1 Expression of FLT3 in normal hematopoiesis.
"+/–" indicates how the expression of FMS-like tyrosine kinase-3 is linked to the process.

a ligand-independent constitutive activation of FLT3 which results in autophosphorylation and phosphorylation of downstream targets. FLT3 receptor consists of an extracellular portion of five immunoglobulin-like domains, a trans-membrane region, a short juxtamembrane unit and the intracellular tyrosine kinase domain. Upon binding the FL, the receptor dimerizes and leads to the phosphorylation of downstream signaling mediated by AKT, MAPK, STAT5 [6] (Fig. 2).

FLT3 ligand and the FLT3 receptor seem to be upregulated in the majority of leukemia cell lines [7, 8]. These mutations result in elevated and constitutive FLT3 activation, which in turn leads to the triggering of STAT5 and downstream MAPK and AKT signaling cascades that causes suppression of apoptosis and dysregulated cell proliferations [9, 10].

The poor prognosis and treatment in AML has lead to the development of effective FLT3 inhibitors as targeted therapy for the patients. Several studies have shown that specific FLT3 inhibitors induce preferential cytotoxicity in FLT3 mutant cells, and that sustained and potent FLT3 inhibition appears essential to bring cytotoxicity against myeloblasts [11, 12].

AC220 is a novel compound expressly optimized as a FLT3 inhibitor for the treatment of AML. In our study we show that AC220 inhibits FLT3 with low potency in cellular assays and is highly selective when

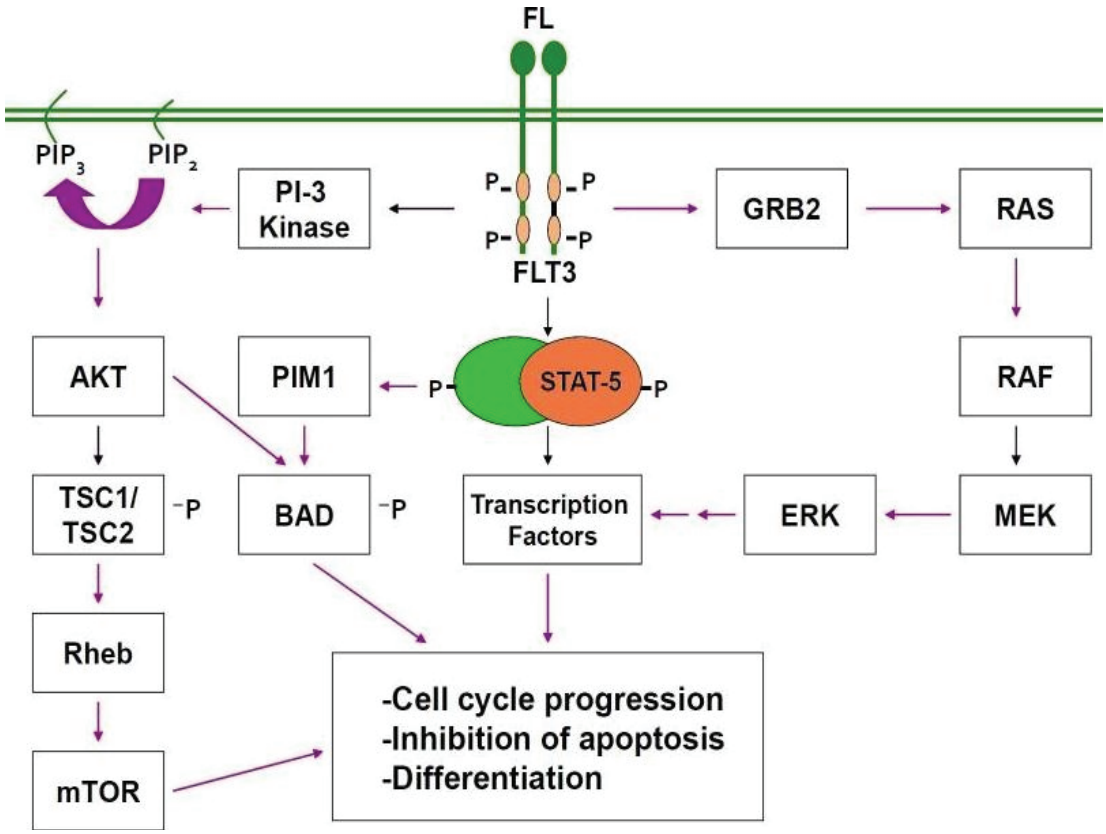

Fig. 2 Signaling cascade diagram of the downstream effects of FLT3 activation in AML.

screened against other proteins. We also show that the combination of AC220 with other compounds like Atorvastatin greatly increases the apoptosis of mutated AML cells whilst preserving the healthy viable cells.

In this study, we show how different combinations of therapeutic drugs inhibit the downstream signaling of mutated FLT3 in MV4-11 cell lines and also show how the inhibition of this signal targets FLT3 mutated AML cells by inhibiting cell survival.

3. Materials and Methods

3.1 Cellular Assays

The MV4-11 human cell line was grown in RPMI 1640 supplemented with 10% fetal bovine serum. For proliferation assays, cells were cultured overnight in low serum media (0.5% FBS), then seeded in a 6-well plate at 100,000 cells per well. Inhibitors were added to the cells and incubated at 37 °C for 24 h. Cell viability was measured using the Cell Titer-Blue Cell Viability Assay from Promega. To measure inhibition of FLT3 autophosphorylation, cells were cultured in low serum media (0.5% FBS) overnight and seeded at a density of 24×10^6 cells per well in a 6-well plate the following day. The cells were incubated with inhibitors for 24 h at 37 °C.

3.2 Apoptosis Assay

MV4-11 cells were diluted to a 2×10^5 cells/mL in RPMI 1640 medium, in the absence of IL-3. Cells were seeded in 12 well plate and the inhibitors were added 24 h at 37 °C for the Atorvastatin and 1 h for AC220, in different concentrations. After the incubation Annexin V was added for staining and the analysis was done by FACS.

4. Results and Discussion.

In order to determine the ability of compounds to inhibit FLT3 in the cellular environment, we measured the inhibition of FLT3 autophosphorylation in the

human leukemia cell lines MV4-11, which harbors a homozygous FLT3-ITD mutation and is FLT3 dependent [13, 14]. Atorvastatin directly disrupts the first step involved in N-linked protein glycosylation, and it was recently demonstrated to inhibit FLT3 glycosylation as well [15]. AC220 was the most potent cellular FLT3-ITD inhibitor. To determine the inhibition of FLT3-ITD signal transduction, we measured MV4-11 cell signaling in the presence of the both inhibitors, combined or alone in different concentrations (Fig. 3).

In order to analyze the effects of AC220 and Atorvastatin, after treating the cells, we used RIPA buffer to lyse the cells and using western blot we tried to understand the pathways involved for the FLT3-ITD signaling. Following the treatment with both compounds we see that the anti-FLT3 antibody produced two bands which represent the glycosylated complex. With the different concentrations we see a change in the glycosylation to the deglycosylated form. Furthermore, phosphorylation of STAT5, ERK and AKT were used as indicators for the activation of STAT5, MAP/ERK and PI3K/AKT pathways and there was also shown a dose-dependency of the p-STAT5 signal. In Fig. 4, we see that the combination of Atorvastatin with AC220 inhibit the FLT3 signaling in a dose-dependent manner with a major inhibition at a combination of 0.5 nM AC220 + 500 μM. These data suggest that Atorvastatin is an inhibitor of the cells that express the aberrant FLT3ITD signal.

The potency of AC220 in blasts observed here is comparable with or better than what has been reported for other FLT3 inhibitors in primary cells [13, 16].

The experiments have shown that Atorvastatin could

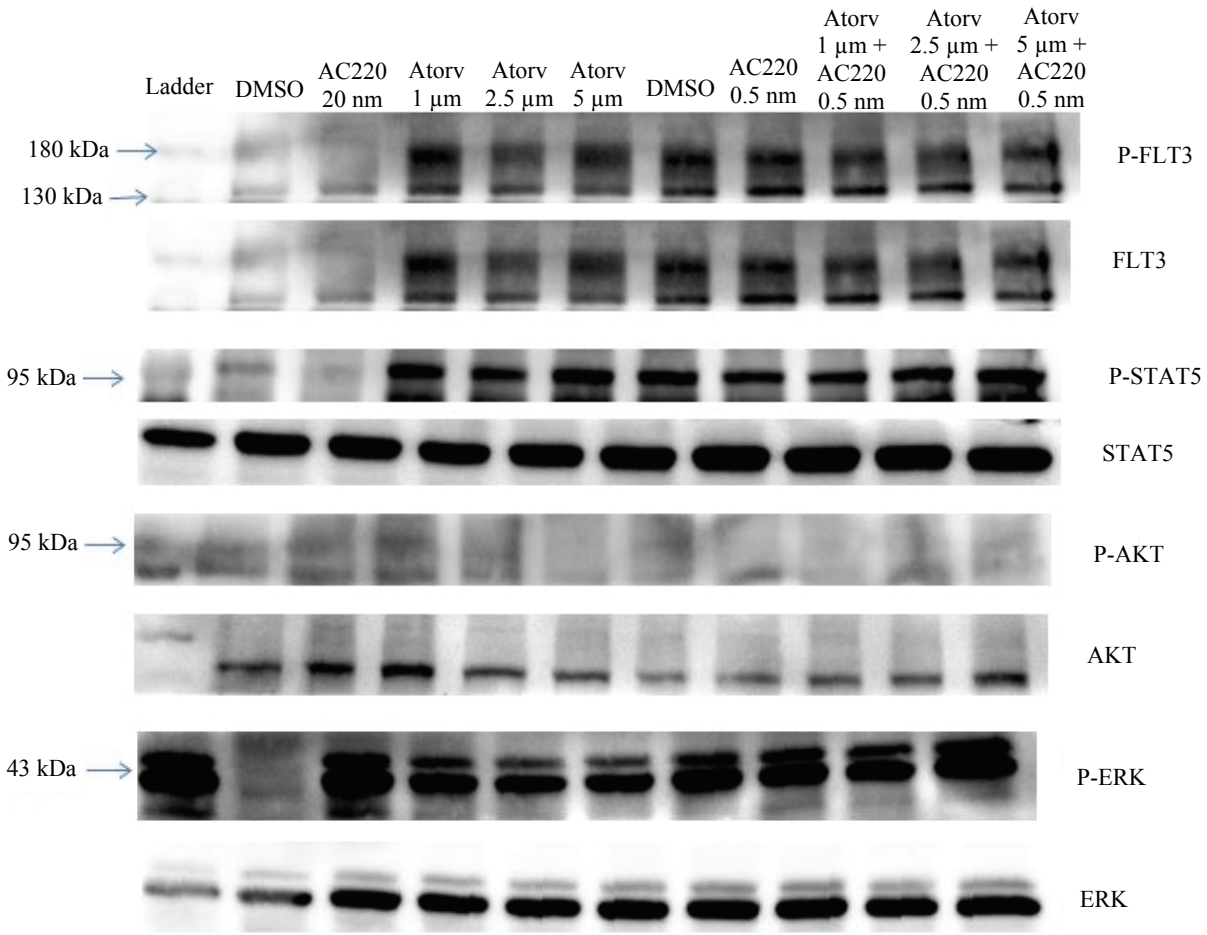

Fig. 3 MV4-11 cell signaling with different concentrations.

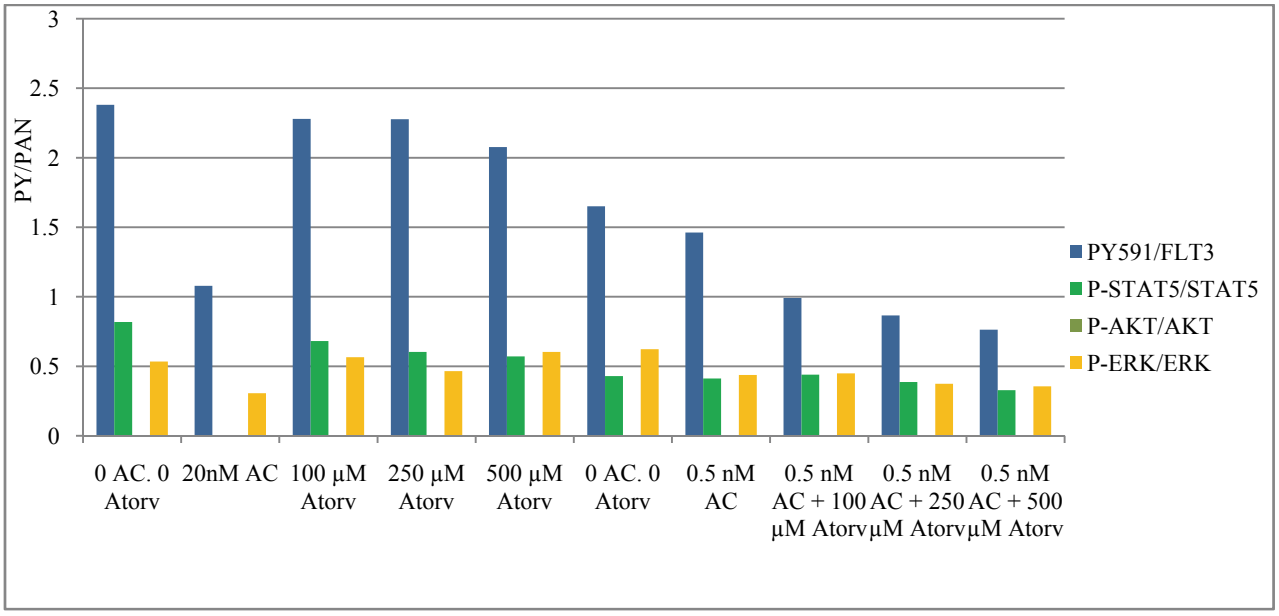

Fig. 4 Quantification datafrom MV4-11 cells treated with Atorvastatin for 24 h and with AC220 for 1 h.

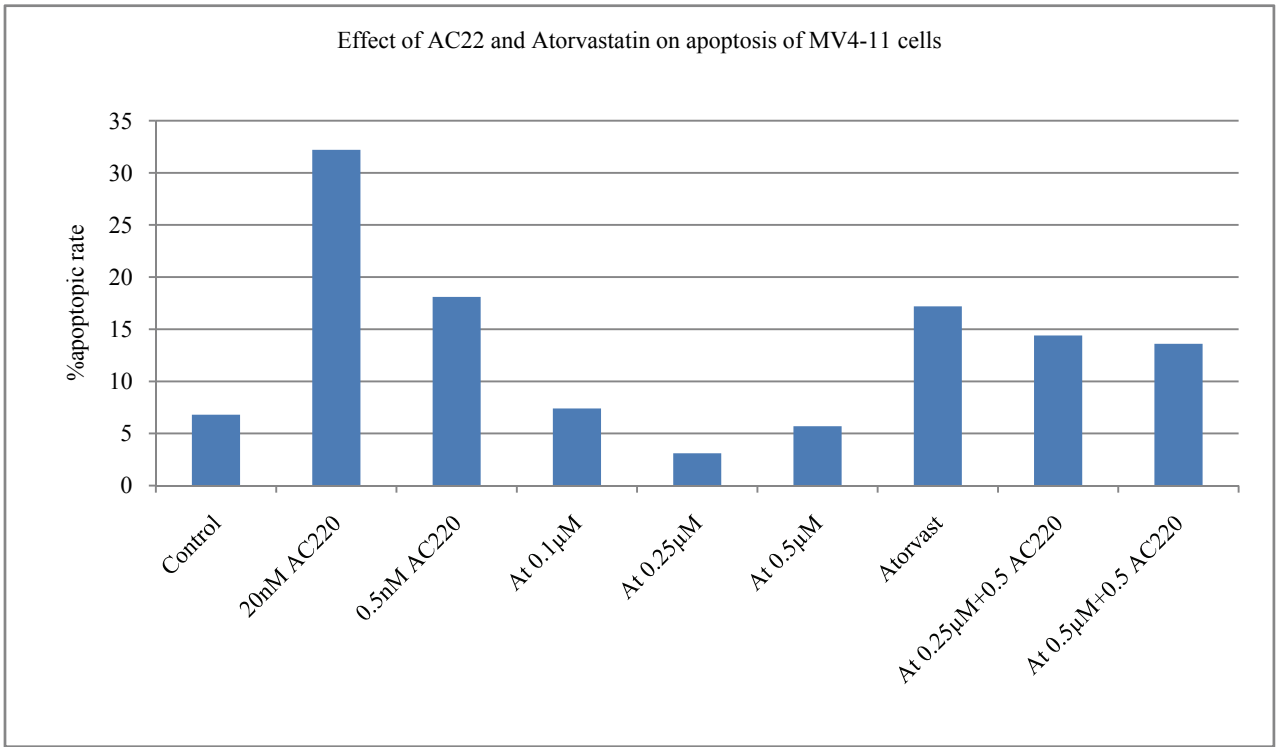

Fig. 5 The viability of MV4-11 cells was defined by MTS assay. The cells were treated for 24 h in the presence of single AC220 or Atorvastatin, or in combination.

enhance the cytotoxic effect of the AC220 on the FLT3ITD leukemia patients. Since Atorvastatin affects the N-glycosylation, we were worried about cytotoxity. Therefore we assessed the effects of Atorvastatin on apoptotic MV4-11 cells to see the effects of the compounds. Effects of AC220 and Atorvastatin were tested on these cells via MTS assays for different concentrations of the drugs and their combinations (Fig. 5). From the table, we observe a high death rate of the cells during the use of single AC220, showing the

high inhibitory effect this drug has. Smaller concentrations of AC220 in combination with different doses of Atorvastatin show good apoptotic effect of the FLT3ITD cells with low percentage of necrotic cells.The potency of AC220 in blasts observed in our experiments is comparable with previous studies for FLT3 inhibitors [13, 17, 18]. The data show that MV4-11 cells are quite sensitive to drug treatment with AC220 and Atorvastatin and that they have a high resistance to the toxicity from AC220.

Acute myeloid leukemia is an aggressive disease with a very poor prognosis and high mortality rate [19, 20]. Inhibiton of FLT3 by Atorvastatin results in growth arrest and apoptosis of MV4-11 cells that express FLT3ITD. These effects are probably the result of blocking the FLT3ITD-mediated activation of many downstream signaling proteins. The proteins aforementioned include ERK1/2, STAT5 and AKT. The activation of STAT5 is a very specific feature to the mutated FLT3 receptors. We found that Atorvastatin inhibits the autophosphrylation of FLT3 in the cells that harbor the FLT3 mutation which shows that the statins family compounds can effectively inhibit the transforming effects of the FLT3 mutation in AML.

5. Conclusions

The association of FLT3 mutations with poor clinical outcomeand the therapeutic effects observed in different studies have shown that treatment with first generation inhibitors implicate FLT3 as an important target for intervention in AML. We present here the role of AC220 as an inhibitor with good potency and specificity regarding FLT3 inhibition. Atorvastatin did not have the same ability as AC220 in regards to inhibition, but together with AC220 we observed a synergistic effect causing a decrease in cell proliferation and apoptosis increase. Unfortunately, Atorvastatin could lower the number of viable cells at the same concentration as AC220. MV4-11 cells were sensitive to the treatment with AC220 and Atorvastatin and could be used for further experiments to find new target therapeutic drugs with better response to FTL3ITD signaling.

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