A Novel Molecular Mechanism of Primary Resistance to FLT3-Kinase Inhibitors in Acute Myeloid Leukemia

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Abstract

Currently, FLT3 tyrosine kinase inhibitors (TKIs) are emerging as the most promising drug therapy to overcome the dismal prognosis of acute myelogenous leukemia (AML) patients harbouring internal tandem duplications (ITD) of FLT3. However, up-front drug resistance occurs in approximately 30% of patients and molecular mechanisms of resistance are poorly understood. Here, we have uncovered a novel mechanism of primary resistance to FLT3 TKIs in AML: a FLT3 receptor harbouring a non-juxtamembrane ITD atypically integrating into the β-2 sheet of the first kinase domain (FLT3_ITD627E) induces dramatic up-regulation of the anti-apoptotic protein MCL-1. Using RNA interference technology, de-regulated MCL-1 protein expression was shown to play a major role in conferring the resistance phenotype of 32D_ITD627E cells. Enhanced and sustained binding of the adaptor protein GRB-2 to the FLT3_ITD627E receptor is involved in MCL-1 upregulation and is independent from TKI (PKC412)-induced inhibition of the receptor kinase. Thus, we describe a new mechanism of primary resistance to TKIs, which operates by reprogramming local and distant signal transduction events of the FLT3 tyrosine kinase. The data presented suggests that particular ITDs of FLT3 may be associated with rewired signaling and differential responsiveness to TKIs.
Introduction:

In 30-40% of adult AML patients, activating mutations in \textit{FMS-like tyrosine kinase 3} (\textit{FLT3}) can be detected and the majority of these mutations belong to the class of internal tandem duplications (ITDs). In AML, ITD mutations of \textit{FLT3} are associated with higher white blood cell counts, an increased relapse rate, and decreased overall survival in response to standard chemotherapy\textsuperscript{1-4}. Therefore, treatment using FLT3 tyrosine kinase inhibitors (TKIs) is a novel and promising approach to overcome the dismal prognosis of FLT3\_ITD-positive AML. Several new small molecule TKIs targeting FLT3 kinase (e.g. PKC412 (midostaurin), CEP-701, SU11248) are currently being investigated in phase II/III clinical trials\textsuperscript{5-7}. These studies have shown so far that monotherapy using FLT3-TKI may result in measurable clinical response including significant reductions of peripheral blood (PB) and bone marrow (BM) blasts. However, in most cases these responses are transient and patients either become resistant to TKI treatment after a short period of response (secondary resistance) or show up-front resistance to FLT3 TKIs (primary resistance)\textsuperscript{5-8}. The kinase inhibitor PKC412 (midostaurin) is a derivative of the alkaloid staurosporine and is described as a potent inhibitor of mutant FLT3 receptors with an IC\textsubscript{50} below 10nM\textsuperscript{9}. In a clinical phase II trial in AML patients using PKC412 monotherapy, of the 28/86 patients with FLT3 mutations identified in the original screening, 20/28 patients (71\%) had a blast response which was defined as >50\% decrease in PB or BM blasts\textsuperscript{10}. Thus, approximately 30\% of patients harbouring FLT3 mutations may exhibit primary resistance to PKC412 treatment\textsuperscript{7,10}.

Up to now, the etiology of primary and secondary resistance to FLT3-TKIs in AML is poorly understood but is of major importance for development of future therapeutic strategies using these compounds. Acquired resistance mutations may prevent TKI binding to the FLT3 receptor as described in resistance to Imatinib mesylate in BCR-
ABL-positive chronic myelogenous leukemia\textsuperscript{11}. Indeed, a limited number of potential resistance mutations surrounding the drug binding site of FLT3 have been predicted by random mutagenesis \textit{in vitro}\textsuperscript{12}. In line with these results, we recently identified a mutation (N676K) in the FLT3 tyrosine kinase domain conferring clinical resistance to PKC412 in FLT3\_ITD-positive AML\textsuperscript{13}. Apart from FLT3 overexpression leading to reduced efficacy of TKIs, activation of compensatory pathways, rendering cells independent of FLT3, has also been proposed as a possible scenario. This idea was further supported by observations that, despite inhibition of the FLT3 kinase by TKIs, downstream pathways remained activated and leukemic blasts proved to be resistant to FLT3-TKIs\textsuperscript{8,14,15}.

In this report, we describe a novel molecular mechanism leading to primary clinical resistance towards TKI-therapy (PKC412) in AML. An atypical ITD-mutation integrating in the first tyrosine kinase domain (TKD 1) at amino-acid position 627 (ITD627E), generating a non-JM ITD\textsuperscript{16}, conferred resistance to a panel of FLT3-TKIs \textit{in vitro} and \textit{in vivo}. Up-regulated expression of myeloid cell leukemia-1 (MCL-1), an anti-apoptotic member of the BCL-2 family, was shown to act as the major resistance mechanism increasing the threshold for induction of apoptosis in response to TKIs.
Material and Methods:

Isolation of primary AML blasts and mutation screening of FLT3_ITD

Heparin-treated PB samples (20ml) were obtained from a patient enrolled in a phase II study investigating the efficacy and toxicity of PKC412 (PKC412A-2104 trial\textsuperscript{,7, 10}) before start of PKC412 treatment, at the timepoint of documented primary resistance (day 35), and 2 days after PKC412 was discontinued after informed consent was obtained in accordance with the Declaration of Helsinki. Laboratory experiments on ITD variants isolated from patient material were performed with approval from the Ethikkomission Mainz institutional review board.

Mononuclear cells (MNC) enriched in AML leukemic blasts were isolated as described\textsuperscript{17}. Genomic DNA from PB MNCs was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). ITD mutation screening by PCR and subcloning of PCR products into pCR4-TOPO vectors (Invitrogen, Groningen, The Netherlands) was performed as described\textsuperscript{16}.

DNA constructs

A human FLT3_ITD construct, subcloned into the pAL expression vector was used and has been previously described\textsuperscript{17}. This ITD allele (36bp / 12aa) integrates between codons 598 and 599 in the JM domain of FLT3. Subcloning of the ITD627E allele into the pAL vector was performed as described\textsuperscript{16}. The ITD627E allele (93bp / 31aa) integrates at codon 627 in the β2-sheet of the TKD1 of FLT3 and leads to an amino acid exchange at codon 627 (alanine to glutamate)\textsuperscript{16}. The FLT3_ITD627A mutant was generated by Medigenomix AG (Martinsried, Germany) by site directed mutagenesis. This ITD mutant is identical in length and position of integration to
ITD627E (93bp / 31aa) with the amino acid at codon 627 reverted to wild type (alanine)
(16). All vector constructs were verified by nucleotide sequencing. The amino acid sequences of the ITD alleles are shown in supplemental figure 1.

**Transfections**

Transfection of 32D cells with different FLT3 DNA-constructs was performed as described (13) and polyclonal cell lines were used for further experiments. All mock-transfected control cells died upon withdrawal of IL-3 from the culture medium. Clonal cell lines from early passages of polyclonal cell lines were generated by limiting dilution in 96 well plates.

32D_ITD mMCL-1 cells, ectopically overexpressing MCL-1, were generated by transfection of 32D_ITD cells with twenty micrograms of p3xFLAG-CMV10 vector containing the coding sequence of murine MCL-1 (kindly provided by Dr. H. Schulze-Bergkamen) by electroporation. Cells were selected with 0.5mg/ml G418 and polyclonal cell lines were used for experiments.

**Protein extract preparation, immunoprecipitation and Western blot analysis**

Isolated AML leukemic blasts and/or 32D transfectants were treated with different concentrations of PKC412 (kindly provided by Novartis, Basel, Switzerland), or U0126 (Cell Signaling Technology, Frankfurt, Germany).

For Western blot analysis (17) the following antibodies were used: anti–phospho-FLT3 (Y591), anti–phospho-AKT (S473), anti–phospho–extracellular signal related kinase 1/2 (ERK1/2) (T202/Y204), AKT, ERK1/2, anti-phospho-S6 protein (S240/S244), S6 protein and anti-phospho-STAT3 (S727 and Y705) (all Cell Signaling Technology, Frankfurt, Germany); FLT3, STAT-5, MCL-1, GRB-2 and STAT3 (Santa Cruz,
Heidelberg, Germany); anti-phospho-tyrosine (4G10) and anti-phospho-STAT5 (Y694/Y699) (Upstate, Lake Placid, NY, U.S.A.); anti-BCL-X_L (BD Biosciences, Heidelberg, Germany); actin (MP Biomedicals, Aurora, OH, U.S.A.), GAPDH (Biosdesign International, Saco, ME, U.S.A) and tubulin (Sigma-Aldrich, Munich, Germany). The FLT3 phosphoepitope-specific antibodies FLT3pY589, FLT3pY599, FLT3pY768, FLT3pY955 and FLT3pY969 were generated in the laboratory of Dr. Rönnstrand. Immunoprecipitation of FLT3 was performed as described with modifications17.

**Apoptosis and viability assays**

The percentage of apoptotic cells was determined by measuring the sub-G1 fraction upon propidium iodide (PI)-incorporation using flow cytometry as described13,18. Further, cellular apoptosis was measured by Annexin V labelling according to the manufacturer’s protocol (BD Biosciences, Heidelberg, Germany). Briefly, 2x10^5 cells/ml were treated with different concentrations of PKC412 for 24h and early and late cellular apoptosis was determined by staining with Annexin V-FITC antibody/propidium-iodide and flow cytometry measurement.

Intact mitochondrial membrane potential was visualized by TMRE (Tetra-methylrhodamine-ether)-staining upon incubation of cells (2.5x10^4 cells/ml) with 50nM TMRE at 37°C for 30min followed by flow cytometry19.

**siRNA experiments**

2x10^6 32D_ITD627E cells were transfected by electroporation with h-FLT3 (siGENOME SMARTpool, M-003137-01; Dharmacon), m-MCL-1 (sc-35878), m-STAT3 (sc-29494), m-BCL-2 (sc-29215) or m-GRB-2 (sc-29335, all Santa Cruz, Heidelberg, Germany) specific siRNAs. siRNAs were dissolved to a final
concentration of 20µM stock solution and for each transfection 10µl siRNA stock solution was used. As a negative control, an equivalent concentration of AllStars Negative Control siRNA (Qiagen, Hilden, Germany) was used. For assessment of apoptosis, cells were left to recover from the transfection for 6h and were then treated with or without PKC412. Percentage of apoptotic cells was assessed after 24 and 48h incubation as described above.

Proteomics

Cellular protein lysates of FLT3_ITD and FLT3_ITD627E 32D cells were prepared as described above using lysis buffer, supplemented with protease and phosphatase inhibitors, supplied by Kinexus Bioinformatics Corp. (Vancouver, Canada). Dye labelling, hybridization and analysis of the Kinex™ antibody array was done by Kinexus.

Autophosphorylation of FLT3 in vitro

32D cells expressing either FLT3_ITD or FLT3_ITD627E, were extracted with lysis buffer. 10µg anti-FLT3 antibody (C-20, Santa Cruz) was added to each extract and incubated at 4°C over night. 30µl protein-G sepharose (Pierce) and 120µl Sepharose CL4B (Sigma) (each 1:1 suspension with lysis buffer) were added. The beads were sedimented and washed three times. Then, the suspension was divided into 8 aliquots, the beads sedimented again and washed once with kinase buffer (20mM Hepes, pH7.5, 5mM MnCl₂, 0.1mM sodium orthovanadate). The liquid was removed carefully and 25µl kinase buffer, and PKC412 in different concentrations were added (final DMSO concentration 1%), and the samples incubated on ice for 15min. The kinase reaction was performed by adding 3-5µCi [³²Pγ] ATP per reaction and subsequent incubation at 30°C for 30min. The reactions were stopped by adding
SDS-PAGE sample buffer, and heating at 95°C for 5min. SDS-PAGE gels were run, subjected to autoradiography and quantitated using a GS250 Molecular Imager (BioRad).

Results

Identification of an atypical FLT3_ITD (FLT3_ITD627E) in an AML-patient with primary resistance to PKC412 treatment.

Clinical material from an AML-patient treated with PKC412 within a phase II clinical trial\textsuperscript{10} was subjected to molecular analysis. Patient characteristics, schedule and dose of PKC412, and hematologic responses have been described previously\textsuperscript{7, 10}. The patient had not experienced a blast response (i.e. > 50% decrease in leukemic blasts) (Fig. 1A) but showed primary resistance to PKC412 (200mg/day)\textsuperscript{10}. RNA and DNA from leukemic blasts were isolated before start of PKC412 therapy and at the timepoint of documented resistance (day 35) (Fig. 1A). At baseline, FLT3 mutation analysis revealed two different ITD alleles (ITD1 and ITD2, Fig 1B), while at the time point of resistance, only one ITD allele was detectable (ITD1, Fig 1B). ITD1 (31 amino acids) integrates at nucleotide 1880 of \textit{FLT3} thereby generating a single nucleotide change from GCA to GAA\textsuperscript{16}. This resulted in an amino acid substitution from alanine to glutamate at amino acid position 627 (ITD627E). An additional mutation in FLT3_ITD627E was excluded. Thus, FLT3_ITD627E represents an atypical ITD of FLT3 not integrating into the JM domain (amino acids 572–603) but into the \(\beta2\)-sheet (amino acids 624–630) of the first kinase domain (TKD1) of FLT3\textsuperscript{16}. Since the ITD627E allele was selected \textit{in vivo} during PKC412 treatment, we next assessed the status of FLT3_ITD627E signaling \textit{ex vivo}. Primary AML blasts from the patient were isolated at the timepoint of resistance. FLT3 protein was found to be
present and phosphorylated in untreated cells (Fig. 1C). Upon incubation with PKC412 at a dose of 10nM and 100nM, inhibition of FLT3 phosphorylation was 64% and 91%, respectively as evaluated by densitometric scanning (Fig. 1C). No residual tyrosine phosphorylation of STAT5 was detectable when cells were treated with 10nM or 100nM PKC412 (Fig. 1C). The degree of PKC412 induced inhibition of FLT3 phosphorylation illustrated in Fig. 1C was comparable to inhibition observed in patients responding to PKC412 (13 and data not shown). Trough levels of PKC412 and of its active metabolite, CGP62221, were obtained at the timepoint of resistance (day 35). The actual level of the sum of PKC412 and CGP62221, corrected for 99% protein binding, was similar (37nM) to trough levels observed in other FLT3_ITD-positive AML patients at time point of complete blast clearance13. Together, this data suggests adequate inhibition of FLT3_ITD627E receptors in vivo at the time of primary resistance.

**FLT3_ITD627E confers cross-resistance towards FLT3-TKIs.**

To assess the biological properties of the FLT3_ITD627E allele in vitro, stably transfected hematopoietic 32D cells expressing the mutant receptor were treated with PKC412 and the percentage of apoptotic cells was determined. Sensitivity of 32D_ITD627E cells was assessed in comparison to 32D cells expressing a standard FLT3_ITD integrating in the JM domain between codons 598 and 59917. In these experiments, 32D_ITD627E proved to exhibit resistance to PKC412 treatment up to concentrations of 130nM (Fig. 2A). As a control, Annexin V/PI assays were also performed with comparable results (data not shown). Analogous results were obtained when TMRE staining and flow cytometry was applied (Fig. 2B). This assay visualizes intact mitochondrial outer membrane potential and its loss is considered a hallmark for apoptosis19. A newly transfected polyclonal cell line harbouring the
ITD627E allele (32D_ITD627Ep4) and two independent clonal cell lines established from early passages of the first polyclonal 32D_ITD627E cell line (32D_ITD627E c1 and 32D_ITD627E c2) showed a similar PKC412-resistant phenotype (supplemental Fig. 2A and 2B).

Next, we addressed the question, whether resistance to PKC412 is solely dependent on the ITD integration site at this particular position or whether the amino acid exchange from alanine to glutamate induced by ITD integration at position 627 is involved. To test this hypothesis, we employed 32D cells stably transfected with a mutant in which the amino acid exchange at position 627 has been reverted to alanine by site directed mutagenesis (32D_ITD627A). 32D_ITD627A cells, 32D_ITD cells, and 32D_ITD627E cells were treated in parallel with PKC412. Again, 32D_ITD627E proved to be resistant to PKC412 treatment whereas 32D_ITD627A cells, like 32D_ITD, showed to be sensitive to PKC412 (Fig. 2C). This result indicates that the point mutation A627E is essential for the resistance phenotype of FLT3_ITD627E.

To determine sensitivity to other FLT3-TKIs, cells were treated with various concentrations of K252a, a compound that is structurally similar to CEP-70112, 20 and with SU5614, a structurally more distant tyrosine kinase inhibitor21, respectively. These experiments demonstrated cross-resistance of 32D_ITD627E cells to these compounds as compared to 32D cells expressing the standard JM-ITD (32D_ITD) (Fig. 2D).

**FLT3 kinase activity is equally down-regulated by PKC412 in FLT3_ITD and FLT3_ITD627E cells; however, activation of ERK1/2 persisted in PKC412 treated FLT3_ITD627E cells.**
To assess the effects of PKC412 on FLT3 signal transduction, 32D_ITD and 32D_ITD627E cells, were exposed to a range of PKC412 concentrations and the activation status of key signaling molecules was determined by Western blotting. Surprisingly, we detected comparable dephosphorylation of the FLT3 receptor at tyrosine 591 (Y591) in both cell lines (Fig. 3A). FLT3-Y591 has been shown to be a major phosphorylation site of activated FLT3 kinase and indicates activated downstream signal transduction\textsuperscript{22}. Furthermore, we found activation of key signaling nodes as STAT5, AKT and S6 protein equally down-regulated by PKC412. Interestingly, this was not the case for ERK1/2 phosphorylated at T202 and Y204, which was up-regulated and remained activated/phosphorylated in 32D_ITD627E cells, as compared to 32D_ITD cells, despite full suppression of FLT3 receptor phosphorylation at Y591 (Fig. 3A).

As a next step immunoprecipitation of the FLT3 receptor followed by immunoblotting was performed using anti-phosphotyrosine-specific antibodies. Consistent with the results described in Fig. 3A, FLT3 was found to be equally dephosphorylated in 32D_ITD and in 32D_ITD627E cells upon treatment with PKC412 (Fig. 3B). To directly determine kinase activity of FLT3_ITD and FLT3_ITD627E receptors, \textit{in vitro} autophosphorylation kinase assays were performed. Fig. 3C illustrates equal inhibition of kinase activity from both FLT3 receptors upon treatment with PKC412.

To analyze the phosphorylation status of the mutant FLT3 receptors in detail, we used a battery of antibodies specific for six different phosphorylation sites of FLT3. Again, we detected equal dephosphorylation at all the different phospho-sites upon PKC412 treatment (Fig. 3D).

To rule out that ERK1/2 phosphorylation and IL3-independent growth of 32D_ITD627E cells had become independent from FLT3 signaling by acquisition of cryptic genetic events we used RNA interference (RNAi) technology. Downregulation
of FLT3_ITD627E by transient transfection of FLT3-specific siRNA led to substantial inhibition of ERK1/2 phosphorylation (31% of control) (Fig. 3E, left panel) and induced apoptotic cell death in a major fraction of 32D_ITD627E cells (Fig. 3E, right panel). These results show that signaling from the mutant FLT3_ITD627E receptor is essential for ERK1/2 phosphorylation and for IL-3 independent survival of 32D_ITD627E cells.

**ERK1/2 activation plays a minor role in mediating resistance to PKC412 in FLT3_ITD627E cells.**

To assess the functional contribution of ERK1/2 activation to the FLT3-TKI resistant phenotype, we applied the highly selective MEK/ERK inhibitor U0126. In these experiments, U0126 completely suppressed ERK1/2 activation in 32D_ITD and in 32D_ITD627E cells (Fig. 4A). Next we tested, whether ERK1/2 inhibition restores PKC412 sensitivity in 32D_ITD627E cells. In comparison to 32D_ITD cells, a small but consistently detectable increase in apoptosis was observed when 32D_ITD627E cells were treated with a combination of PKC412 and the ERK1/2 inhibitor U0126 for 48h (Fig. 4B). This suggests that ERK activation may have a minor contribution to the TKI-resistance phenotype of FLT3_ITD627E but does not play an essential role.

**MCL-1 protein expression is up-regulated in 32D_ITD627E cells and suppression of MCL-1 rescues TKI-sensitivity.**

To screen 32D_ITD627E cells for differentially activated signaling pathways, we performed protein expression and phosphorylation profiling. The Kinex™ antibody microarray allows comparison of protein expression and of the phosphorylation status of 615 different proteins. Using this platform, we could identify 62 differentially expressed/phosphorylated proteins in protein lysates from FLT3_ITD627E cells
compared to FLT3_ITD cells (Fig. 5A and Table 1). A major proportion of these proteins is known to be involved in signal transduction. Our next step was to validate these findings and to determine the functional role of these proteins. Table 1 shows that serine phosphorylation of STAT3 at position S727 was found to be up-regulated by 50% in comparison to control. Immunoblot analysis indeed demonstrated an increased level of STAT3 S727-phosphorylation as compared to 32D_ITD cells (Fig. 5B, upper panel). Full activation of STAT3 is known to require serine phosphorylation at position 727 in addition to tyrosine phosphorylation\textsuperscript{24}. RNA interference experiments using FLT3-specific siRNA confirmed that FLT3_ITD627E receptor is essential for up-regulation of serine phosphorylation of STAT3 at position 727 (Fig. 5B, lower panel). To determine whether increased P-S727-STAT3 signaling underlies the resistance phenotype of 32D_ITD627E cells, STAT3 protein expression was down-regulated by STAT3-specific siRNA and sensitivity to PKC412 was measured. Fig. 5C shows that suppression of STAT3 restored sensitivity to PKC412 in a minor proportion of cells. This result suggests that signaling via STAT3 contributes to the resistance phenotype of 32D_ITD627E cells but does not appear to play a major role. This result is also in line with the observation that phosphorylation of STAT3 at S727 and also Y705 was downregulated upon PKC412 treatment (supplemental figure 4A and 4B).

Strikingly, by antibody microarray analysis, MCL-1 (myeloid cell leukemia differentiation protein 1) was found to be the most up-regulated protein in 32D_ITD627E cells (+675%; Table 1). Western blotting confirmed this finding (Fig. 5D (upper panel) and Supplemental Fig. 2A and 2B). Interestingly, in 32D_ITD627E cells, MCL-1 expression was unchanged upon PKC412-treatment, while in 32D_ITD cells PKC412 treatment resulted in a marked decrease of MCL-1 expression with regard to the higher molecular weight subspecies (Fig. 5D, upper panel). Appearance
of MCL-1 as a doublet in SDS-PAGE analysis has been described and has been attributed to differentially phosphorylated isoforms\textsuperscript{25}.

Confirmation that MCL-1 expression is indeed regulated by the FLT3\_ITD627E receptor was achieved by siRNA experiments. Figure 5D (lower panel) shows that knock-down of FLT3\_ITD627E using FLT3-specific siRNA resulted in significant suppression of MCL-1 protein levels. However, FLT3\_ITD627E regulated MCL-1 expression does not appear to involve the ERK1/2 or STAT3 pathways because neither suppression of phosphorylated ERK1/2 by the selective MEK/ERK inhibitor U0126 nor suppression of STAT3 by RNA interference resulted in decreased MCL-1 protein levels (data not shown). Interestingly, stable MCL-1 protein expression, despite FLT3 receptor dephosphorylation, could also be demonstrated in primary AML blasts isolated from the patient and treated \textit{ex vivo} with PKC412 (supplemental Fig. 3B).

Finally, we tested whether MCL-1 up-regulation contributes to the resistance phenotype of 32D\_ITD627E cells. By decreasing MCL-1 protein levels using MCL-1-specific siRNA, the majority of 32D\_ITD627E cells proved to restore sensitivity to PKC412 (10nM) treatment (Fig. 5E, right panel). Induction of apoptosis was further enhanced by increasing the dose of PKC412 (20nM) indicating rescue of dose-dependent sensitivity to PKC412. From this data it appears that de-regulated MCL-1 expression acts a major resistance factor in 32D\_ITD627E cells. To exclude off-target effects on related antiapoptotic proteins, we controlled for expression of BCL-2 and BCL-X\textsubscript{L} protein in MCL-1-siRNA transfected cells and found both protein levels to be unchanged (Fig. 5E, left panel). As differences in siRNA knockdown efficiencies in favour of MCL-1 might account for the pronounced effect observed upon MCL-1 downregulation but not STAT3 knockdown, we determined the level of suppression...
using densitometric scanning. Mean suppression levels of STAT3 were consistently higher (40.4%) than those of MCL-1 (27.5%) (data not shown).

The unique role of MCL-1 in mediating PKC412-resistance is further supported by the fact that downregulation of the antiapoptotic BCL-2 protein only had a minor effect on PKC412 resistance. Transient transfection of 32D_ITD627E cells with BCL-2-specific siRNA caused a moderate increase in apoptotic cell death (Fig. 5F), comparable to those seen in STAT3 siRNA knockdown experiments (Fig. 5C). Finally, ectopic overexpression of murine MCL-1 in 32D_ITD cells alone was sufficient to confer a PKC412-resistant phenotype, similar to that observed in 32D_ITD 627E cells (Fig. 5G). Expression levels of other anti-apoptotic proteins, like BCL-2 and BCL-XL, were unaffected by overexpression of murine MCL-1 in 32D_ITD cells (supplemental Fig. 4C).

In contrast to 32D_ITD627E cells, only minimal STAT3-S727-phosphorylation and MCL-1 expression was detected in 32D_JM_ITD cells (Fig 5B, top and 5D, respectively). In addition, MCL-1 expression is clearly dependent on FLT3-autophosphorylation in 32D_JM_ITD cells as shown in Figure 5D. To further explore these differences in signal transduction, we transfected 32D_JM_ITD cells with siRNA targeting either STAT3 or MCL-1 and treated the cells with PKC412. Whereas knockdown of MCL-1 causes, similar to FLT3_ITD627E expressing cells, increased sensitivity to PKC412 treatment, the same effect was observed upon suppression of STAT3 (supplemental Fig. 3A) and GRB-2 (data not shown). Surprisingly, no effect on MCL-1 expression was seen upon knockdown of FLT3 using specific siRNA (supplemental Fig. 3C). However, as FLT3 knockdown was not perfect, residual FLT3 receptors are likely still constitutively phosphorylated and mediate persistent MCL-1 expression.
GRB-2/FLT3_ITD627E receptor interaction is maintained upon treatment with PKC412 and is essential for up-regulation of MCL-1 and for the TKI-resistance phenotype.

We next aimed to identify a signaling intermediate which persisted in an active state despite PKC412-induced dephosphorylation of the FLT3_ITD627E receptor and which may represent a link to MCL-1 overexpression. GRB-2 appeared to be an interesting candidate since it is known to participate in FLT3 signaling and in regulation of the MEK/ERK pathway\textsuperscript{26, 27}. To determine binding of GRB-2 to the FLT3-receptor, we treated 32D_ITD, 32D_ITD627E and 32D_ITD627A cells, with PKC412 (10nM), immunoprecipitated FLT3 from cellular lysates and analyzed co-immunoprecipitated GRB-2 by Western blotting. In untreated cells, significantly higher levels of GRB-2 protein bound to the FLT3_ITD627E receptor were found compared to FLT3_ITD and FLT3_ITD627A receptors (Fig. 6A, upper panel). This difference was not due to up-regulation of total GRB-2 protein expression as in all cell lysates equal amounts of GRB-2 protein were detected (Fig 6A, lower panel). Therefore, the interaction of GRB-2 with the FLT3 receptor was disrupted in PKC412-sensitive 32D_ITD and 32D_ITD627A cells, but persisted in PKC412 resistant 32D_FLT3_ITD627E cells (Fig. 6A). Furthermore, sustained association of GRB-2 with the FLT3_ITD627E receptor was still detected in cells treated with PKC412 concentrations as high as 50nM (supplemental Fig. 3D). Binding of GRB-2 to the FLT3 receptor has been described to serve as platform for activation of a variety of signaling pathways. Thus, it is conceivable that persistant binding of GRB-2 to the FLT3_ITD627E receptor accounts for tyrosine phosphorylation-independent activation of a signaling node involved in up-regulation of MCL-1.

The functional relationship between GRB-2, MCL-1 and the PKC412-resistance phenotype was assessed by downregulation of GRB-2 using GRB-2-specific siRNA.
Figure 6B illustrates that knockdown of GRB-2 results in decreased MCL-1 expression. When GRB-2 siRNA-transfected cells were treated with PKC412, sensitivity to PKC412 was restored in the majority of previously resistant 32D_ITD627E cells (Fig. 6B). These data are consistent with results presented above (Fig. 5E) and together suggest that in 32D_ITD627E cells, persistent binding of GRB-2 mediates MCL-1 over-expression and resistance to FLT3-TKIs.

Discussion

In this report we describe a novel mechanism of resistance to FLT3 tyrosine kinase inhibitors: in clinical material derived from an AML patient showing primary resistance to PKC412 we identified an unique non-JM ITD FLT3 receptor featuring ITD integration in the first tyrosine kinase domain (TKD 1) at amino acid position 627 (FLT3_ITD627E). While two different FLT3_ITD alleles were present before start of PKC412, re-evaluation at the time of clinical resistance showed clonal evolution of FLT3_ITD627E-positive blasts only. Thus, it appears that upon start of PKC412 treatment rapid selection of a drug-resistant leukemic clone harbouring FLT3_ITD627E had occurred. This hypothesis was confirmed by testing FLT3_ITD627E in a reconstitution model. These experiments demonstrated that FLT3_ITD627E is sufficient to confer resistance to a panel of FLT3-TKIs in vitro. To systematically examine the molecular basis of TKI-resistance, an antibody microarray screen was applied. This screen identified the anti-apoptotic protein MCL-1 to be dramatically up-regulated in FLT3_ITD627E cells. Functional validation using RNA interference showed that MCL-1 up-regulation was strictly dependent on expression of FLT3_ITD627E receptors and that suppression of MCL-1 levels rescued sensitivity
to PKC412. The anti-apoptotic effects of MCL-1 up-regulation in FLT3_ITD627E cells are currently unknown and are subject for further studies. However, MCL-1 may prevent cytochrome c-release from mitochondria by blocking proapoptotic members of the BCL-2 protein family, thereby impeding their activation and mitochondrial outer membrane permeabilization (MOMP). Our data using TMRE staining is consistent with the concept that up-regulated MCL-1 expression protected from PKC412-induced MOMP. In line with these results, elevated MCL-1 expression has been identified in various human cancers and is associated with poor prognosis and drug resistance29-33.

Kinase measurements demonstrated that FLT3 tyrosine kinase activity of both the FLT3_ITD627E receptor and the FLT3_JM_ITD receptor was equally inhibited upon incubation with PKC412. FLT3 tyrosine phosphorylation was investigated applying 4 different approaches and as compared to 32D_JM_ITD, no significant differences were detected upon PKC412 treatment. Accordingly, with respect to the mechanism, this result shows that resistance to PKC412 is not associated with failure to effectively inhibit FLT3 kinase activity. Interestingly, this is consistent with results obtained in clinical material: Analysis of FLT3-signaling in primary AML blasts isolated at the time point of resistance exhibited effective inhibition of FLT3-kinase upon ex vivo treatment with PKC412. These results point to a FLT3-independent mode of resistance. However, our data show that downstream signaling nodes of FLT3_ITD627E are not uniformly inhibited by PKC412. Using a panel of Western blot analyses, phosphorylation of ERK1/2 and expression of MCL-1 protein proved to be independent from PKC412-induced dephosphorylation of the FLT3_ITD627E receptor. To investigate this discrepancy, we studied interaction of the FLT3_ITD627E receptor with the adaptor GRB-2, situated up-stream of MEK/ERK and MCL-1, with surprising results. Although PKC412 induced dephosphorylation of
the FLT3_ITD627E receptor, GRB-2-binding was maintained at high levels. Phosphorylation independent binding of GRB-2 to FLT3_ITD627E may be accomplished by the N-terminal SH3 domain or the C-terminal SH3 domain of GRB-2. A possible alternative mechanism would be phosphorylation-independent binding of GRB-2 via the SH2 domain. This hypothesis is supported by published data demonstrating that phosphorylation is required for binding of most SH2 domains, however, SH2 domains including GRB2-SH2 domains may bind to their peptide recognition domains in the absence of phosphorylation, albeit with lower affinity\textsuperscript{34-37}. Interestingly, the analysis of single FLT3 phosphoepitopes (Fig. 3D) included 3 potential GRB2 binding sites (Y955, Y969 and Y768), also suggesting a phosphorylation independent association of FLT3_ITD627E with GRB2. Although, the precise mechanism involved in GRB-2/FLT3_ITD627E interaction currently is unknown and residual phosphorylation at yet undetected amino acid residues can not be completely ruled out, our data are consistent with the concept that a neo-binding-motif is generated by integration of ITD627E. This neo-motif appears to critically depend on the amino acid exchange of alanine to glutamate at position 627 since reverting glutamate to alanine abrogated both increased and phosphorylation-independent binding of GRB-2. It also seems to be important that this amino acid exchange was caused by the ITD integration at position 627 in the tyrosine kinase domain 1 of FLT3. We have recently reported that 28.7% of all FLT3_ITD positive patients harbour an integration in the TKD1 of FLT3 and not the juxtamembrane domain of the receptor\textsuperscript{16}. It is tempting to speculate that amino acid exchanges induced by ITD integrations in this highly conserved region are more likely to have dramatic consequences for the overall structure of the FLT3 receptor than integrations in the JM domain.
Comparable FLT3 tyrosine kinase activity was noted in extracts from ITD627E and JM_ITD cells. Accordingly, as to the mechanism of MCL-1 up-regulation, a difference in kinase activity is unlikely. However, increased association of GRB-2 with the FLT3_ITD627E receptor has been detected and GRB-2 was shown to be essential for MCL-1 expression and for the TKI-resistance phenotype. Thus, we propose the following molecular mechanism: (1) generation/exposure of a neo-epitope by integration of ITD627E into the FLT3 receptor serves as a platform for enhanced binding of GRB-2 to the receptor; (2) signals originating from differential binding of GRB-2 promote up-regulation of MCL-1 expression; (3) GRB-2 binding to the FLT3_ITD627E receptor and MCL-1 expression is sustained upon inhibition of the FLT3-kinase by TKIs and this results in an increased threshold for induction of apoptosis in response to FLT3-TKIs.

In addition to MCL-1, a number of signaling molecules as ERK1/2 and STAT3 were also shown to be de-regulated in FLT3_ITD627E cells. However, functional analysis employing selective inhibitors and RNA interference, indicated that ERK1/2 and STAT3 may play a minor role in TKI resistance and suggests that up-regulation of MCL-1 accounts for most of the FLT3_ITD627E-induced resistance phenotype.

Interestingly, in primary AML blasts from FLT3-TKI resistant cases, it has been observed previously that MAP kinase activation may persist despite complete inhibition of FLT3. This has been attributed to FLT3 independent survival pathways. However, in our model, sustained activation of ERK1/2 was shown to strictly depend on FLT3 signaling as revealed by RNA interference assays. Thus, our data suggest that in TKI-resistant primary AML blasts, both FLT3-independent and FLT3-dependent mechanisms may underlie persistent MAP kinase activation.

In conclusion, in this report, we have uncovered the functional role of an atypical ITD integrating into the β2-sheet of FLT3 for drug resistance to TKIs in vivo and in vitro.
The data presented suggests that particular ITDs of FLT3 may be associated with rewired signaling and differential responsiveness to TKIs. We believe, it will be interesting to extract this information in the context of clinical trials employing FLT3-TKIs.

**Acknowledgements:**

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F.B. designed experiments, performed research, analyzed data and participated in writing the manuscript; B.M. designed experiments, performed research and analyzed data; S.K. performed experiments and analyzed data; B.C. and T.S. performed research; F.D.B. designed experiments, performed research and analyzed data; K.M and L.R. performed research and analyzed data; C.H. contributed to interpretation of experimental data; T.K. analyzed data and wrote the manuscript; T.F. designed and supervised experimental work, analyzed data and wrote the manuscript.

Conflict of Interest disclosure: T.F. received honoraria from Novartis.

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References:


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Legends:

Fig. 1: Primary clinical resistance to PKC412 therapy in an AML patient with FLT3_ITD627E

(A): Serial counts of white blood cells (WBC) and peripheral leukemic blasts at several time points during PKC412-therapy and at the time point of clinical resistance (day 35) are depicted.

(B): Results of FLT3_ITD screening by PCR using genomic DNA from primary AML blasts before PKC412 therapy (baseline (BL)) and at the time point of primary resistance (progressive disease (PD)) at day 35 of PKC412 therapy. ITD1 and ITD2 indicate two different FLT3_ITD alleles. wt refers to wild type FLT3.

(C): Primary AML blasts isolated at the time of primary resistance (2 days after PKC412 had been discontinued), harbouring the FLT3_ITD627E allele (A), were treated \textit{ex vivo} with different concentrations of PKC412 for 1h. Tyrosine phosphorylation of FLT3 and STAT5 was analyzed by immunoblotting of cellular lysates.

Fig. 2: FLT3_ITD627E is sufficient to confer resistance to PKC412 in a reconstitution model

(A): 32D_ITD cells (black bars) and 32D_ITD627E cells (light grey bars) were treated with increasing concentrations of PKC412 for 24h and 48h and the percentage of apoptotic cells was measured as subG1 DNA content by flow cytometry.

(B): Intact mitochondrial outer membrane potential of 32D_ITD (black bars) and 32D_ITD627E cells (light grey bars), respectively was assessed by flow cytometric measurement of TMRE–positive cells upon incubation with a range of PKC412
concentrations. Fig. 2B illustrates that the FLT3_ITD627E receptor prevents PKC412-induced mitochondrial outer membrane permeabilization. 

(C): The percentage of apoptotic cells corresponding to subG1 DNA content was determined by flow cytometry after treatment of 32D_ITD (black bars), 32D_ITD627E (light grey bars) and 32D_ITD627A (dark grey bars) with PKC412 for 24h and 48h. In each figure, means of at least three independent experiments are depicted. Error bars represent mean +/- standard deviation. 

(D): 32D_ITD (black bars) and 32D_ITD627E (light grey bars) cells were treated for 48h with various concentrations of K252a (left panel) or SU5614 (right panel) and the percentage of apoptotic cells was determined by flow cytometry. Shown are the means of at least three independent experiments. Error bars represent mean +/- standard deviation. 

Fig. 3: Inhibition of FLT3 downstream signaling nodes and of FLT3 kinase activity by PKC412 in 32D_ITD627E cells and in 32D_ITD cells. 

(A): 32D_ITD and 32D_ITD627E cells were treated with or without different concentrations of PKC412 for 2h. Protein expression and phosphorylation of FLT3 (Y591), STAT5 (Y694/Y699), AKT (S473), ERK1/2 (T202/Y204), and S6 protein (S240/S244), respectively was determined by immunoblotting. 

(B): Overall tyrosine phosphorylation of FLT3 in 32D_ITD and 32D_ITD627E cells after treatment with PKC412 for 2h was determined by immunoprecipitation of FLT3 and Western blot analysis using anti-phospho-tyrosine antibody (4G10). 

(C): Assessment of in vitro kinase activity of immunoprecipitated FLT3_ITD and FLT3_ITD627E receptors, respectively upon incubation in absence and in presence
of various concentrations of PKC412. The experiment shown is representative of 4 with consistent results.

(D): Overall tyrosine phosphorylation and tyrosine phosphorylation of specific phosphoepitopes of FLT3 in 32D_ITD and 32D_ITD627E cells after treatment with PKC412 for 2h was determined by immunoprecipitation of FLT3 and Western blot analysis using anti-FLT3 antibody, anti-phospho-tyrosine antibody (4G10), anti-FLT3pY591, anti-FLT3pY589, anti-FLT3pY599, anti-FLT3pY768, anti-FLT3pY955 and anti-FLT3pY969 antibodies.

(E): Phosphorylation of ERK1/2 (T202/Y204) was assessed in 32D_ITD627E cells by immunoblotting 24h after introduction of FLT3-specific siRNA (left panel). Percentage of apoptotic 32D_ITD627E cells was determined in parallel by flow cytometry 24h and 48h after siRNA knockdown of FLT3 (right panel).

**Fig. 4: ERK1/2 inhibition and sensitivity to PKC412 in FLT3_ITD627E cells**

(A): Phosphorylation of ERK1/2 (T202/Y204) was analyzed by Western blotting in 32D_ITD and 32D_ITD627E cells after treatment for 2h with and without U0126.

(B): Induction of apoptosis was assessed in 32D_ITD (black bars) and 32D_ITD627E (light grey bars) cells, respectively after treatment with PKC412 (10nM), U0126 (10µM) or with a combination of both for 24h (left panel) and 48h (right panel). Shown is one representative result out of a total of three.

**Fig. 5: Functional role of S727-STAT3 and of MCL-1 up-regulation for resistance to PKC412.**

(A): Antibody array analyzing expression and/or phosphorylation of 615 proteins in duplicates using lysates from 32D_ITD and 32D_ITD627E cells. Data obtained from
the antibody array was filtered as follows: (1) Flag 0. This means spot quality is acceptable based on morphology and background; (2) change from control (CFC) >50% or <-50% CFC. The percentage change from control is a measure of the change in normalized signal intensity averages between the experimental sample (FLT3_ITD627E) and the control sample (FLT3_ITD); (3) total error range: <30%. The total error range is the sum of “% error range” from the experimental and control samples.

(B): Phosphorylation of STAT3 at S727 in 32D_ITD and 32D_ITD627E cells, respectively, was determined by immunoblotting (upper panel). In FLT3_ITD627E cells, introduction of FLT3-specific siRNA results in downregulation of P-S727-STAT3 levels while protein levels of STAT3 remain unchanged (lower panel). FLT3 expression and phosphorylation of STAT3 at S727 was assessed in 32D_ITD627E cells by immunoblotting 24h after siRNA mediated knockdown of FLT3.

(C): Suppression of STAT3 expression by RNA interference increases sensitivity of FLT3_ITD627E cells to PKC412. The percentage of apoptotic 32D_ITD627E cells was determined by flow cytometry 48h after introducing STAT3-specific siRNA and treatment of cells with and without PKC412, respectively (right panel). As a control, down-regulation of STAT3 expression was assessed at 24h by immunoblotting (left panel). Shown are the results of one representative experiment out of a total of two.

(D): MCL-1 is up-regulated in 32D_ITD627E cells. Protein expression of MCL-1 in cellular lysates of 32D_ITD and 32D_ITD627E cells treated with and without different concentrations of PKC412 (2h) was determined by immunoblotting (upper panel). Arrows indicate subspecies of MCL-1 as detected by Western blot analysis. In FLT3_ITD627E cells, introduction of FLT3-specific siRNA results in downregulation of MCL-1 levels while protein levels of STAT3 remain unchanged (lower panel). Expression of FLT3, MCL-1 and STAT3, respectively was assessed in 32D_ITD627E...
cells at 24h by immunoblotting. Results shown correspond to one representative experiment (partially depicted in Fig. 3E (left panel)) out of a total of two.

(E): Suppression of MCL-1 expression by RNA interference rescues sensitivity of FLT3_ITD627E cells to PKC412. Induction of apoptosis was determined by flow cytometry 48h after introduction of *MCL-1*-specific siRNA in response to incubation with and without PKC412 (10nM and 20 nM) for 48h (right panel). As a control, protein expression of MCL-1, BCL-X<sub>L</sub> and of BCL-2 in 32D_ITD627E cells was assessed by immunoblotting at 24h (left panel). Results of one representative experiment out of a total of two are shown.

(F): Suppression of BCL-2 expression by transient siRNA transfection. Induction of apoptosis was determined by flow cytometry 48h after introduction of *BCL-2*-specific siRNA in response to incubation with and without PKC412 (5nM and 10 nM) for 48h (right panel). As a control, protein expression of BCL-2 and of MCL-1 in 32D_ITD627E cells was assessed by immunoblotting at 24h (left panel). Results of one representative experiment out of a total of two are shown.

(G): Ectopic expression of FLAG-tagged murine MCL-1 (FLAG-mMCL-1) in 32D_ITD cells confers resistance to PKC412. Protein expression of FLAG-mMCL-1 in 32D_ITDmMCL-1 cells was determined by immunoblotting using an anti-FLAG antibody and actin was used as loading control (left panel). Percentage of apoptotic cells was assessed by flow cytometric measurement of subG1 DNA content in 32D_ITD and 32D_ITDmMCL-1 cells after incubation with PKC412 for 24h (right panel). The means of three independent experiments are shown and error bars represent mean +/- standard deviation.

**Fig. 6: GRB-2/FLT3_ITD627E interaction is sustained upon dephosphorylation of FLT3 by PKC412.**
(A): Analysis of GRB-2/FLT3_ITD627E interaction upon incubation with PKC412. FLT3 was immunoprecipitated from protein lysates of 32D_ITD, 32D_ITD627E and 32D_ITD627A cells after treatment with 10nM PKC412 for 2h and the amount of co-immunoprecipitated GRB-2 was assessed by immunoblot analysis. As a control, GRB-2 protein expression was determined in lysates (100 μg) from each cell line.

(B): Suppression of GRB-2 protein expression by RNA interference rescues sensitivity of FLT3_ITD627E cells to PKC412. The percentage of apoptotic 32D_ITD627E cells was assessed by flow cytometry 48h after introduction of GRB-2-specific siRNA and treatment of cells with and without PKC412 (right panel). GRB-2 and MCL-1 expression was controlled at 48h by immunoblotting (left panel). Results from one representative experiment out of a total of two are shown.

Table 1: Results of antibody microarray screening.

Depicted is a list of differentially regulated proteins/phosphoproteins as detected in 32D_ITD627E cells. Shown are proteins with a more than 50% change in expression/phosphorylation as compared to 32D_ITD control cells (% CFC = % change from control).
### Table 1

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Figure 1

A

![Graph showing cell count by day of therapy with bars for WBC and BLASTS.

- Day 1: WBC 30,000, BLASTS 20,000
- Day 8: WBC 20,000, BLASTS 15,000
- Day 15: WBC 15,000, BLASTS 10,000
- Day 35: WBC 50,000, BLASTS 30,000]
Figure 1
Figure 1

PKC412 1h [nM]  0  10  100

p-FLT3
FLT3
p-STAT5
STAT5
actin
Figure 2

A

24h PKC412

48h PKC412
Figure 2

B
Figure 2
Figure 2

48h K252a

48h SU5614
Figure 3

| PKC412 2h [nM] | 0 | 5 | 10 | 50 | 100 | 0 | 5 | 10 | 50 | 100 |
|----------------|---|---|----|----|-----|---|---|----|----|----|-----|
| p-FLT3         |   |   |    |    |     |   |   |    |    |    |     |
| FLT3           |   |   |    |    |     |   |   |    |    |    |     |
| p-STAT5        |   |   |    |    |     |   |   |    |    |    |     |
| STAT5          |   |   |    |    |     |   |   |    |    |    |     |
| p-AKT          |   |   |    |    |     |   |   |    |    |    |     |
| AKT            |   |   |    |    |     |   |   |    |    |    |     |
| p-ERK          |   |   |    |    |     |   |   |    |    |    |     |
| ERK            |   |   |    |    |     |   |   |    |    |    |     |
| p-S6p          |   |   |    |    |     |   |   |    |    |    |     |
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### Figure 3

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</table>
Figure 3

- Unspecific siRNA
- FLT3 siRNA

FLT3

p-ERK

ERK

Bar chart showing % apoptosis at 24h and 48h:
- Unspecific siRNA
- FLT3 siRNA
Figure 4

A 32D_ITD  32D_ITD627E

<table>
<thead>
<tr>
<th></th>
<th>Med</th>
<th>10μM</th>
<th>Med</th>
<th>10μM</th>
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p-ERK

ERK

actin
Figure 4
Figure 5

A
Figure 5

C

unspecif. siRNA  STAT3 siRNA

STAT3

GAPDH

% apoptosis

48h unspecif. siRNA  48h STAT3 siRNA

Ctrl.
10nMPKC412
Figure 5

<table>
<thead>
<tr>
<th>PKC412 2h [nM]</th>
<th>32D_ITD</th>
<th>32D_ITD627E</th>
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- MCL-1
- actin
- unspecif. FLT3 siRNA
- FLT3
- MCL-1
- STAT3
Figure 5

[Graph showing % apoptosis for unspecified siRNA and MCL-1 siRNA treatments with different concentrations of PKC412.]
Figure 5

F

unspecif. BCL-2 siRNA

Bcl-2
Mcl-1
actin

% apoptosis

0 10 20 30 40 50 60 70 80 90 100

Med 5nM PKC412 10nM PKC412

32D.ITD627E unspecif. siRNA
32D.ITD627E BCL-2 siRNA
Figure 5

G

**32D.ITD**  32D.ITD
mMCL-1

**FLAG-mMCL-1**

**actin**

![Graph showing % apoptosis vs. concentration of 32D.ITD and 32D.ITD mMCL-1](graph.png)

- X-axis: Med, 10nM, 20nM, 50nM, 80nM, 100nM
- Y-axis: % apoptosis

Legend:
- 32D.ITD
- 32D.ITD mMCL-1
Figure 6

A

<table>
<thead>
<tr>
<th>PKC412 2h [nM]</th>
<th>32D_ITD 0</th>
<th>32D_ITD627E 0</th>
<th>32D_ITD627A 0</th>
<th>32D_ITD 10</th>
<th>32D_ITD627E 10</th>
<th>32D_ITD627A 10</th>
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<td>GRB-2</td>
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32D_ITD 627E 627A

GRB-2 (lysate)
Figure 6

B

<table>
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<th>unspecif. siRNA</th>
<th>GRB-2 siRNA</th>
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</thead>
<tbody>
<tr>
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<td>MCL-1</td>
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<tr>
<td>GAPDH</td>
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</tbody>
</table>

% apoptosis

48h unspecif. siRNA 48h GRB2 siRNA

- Ctrl.
- 10nM PKC412
A novel molecular mechanism of primary resistance to FLT3-kinase inhibitors in acute myeloid leukemia

Frank Breitenbuecher, Boyka Markova, Stefan Kasper, Birgit Carius, Torsten Stauder, Frank D. Bohmer, Kristina Masson, Lars Ronnstrand, Christoph Huber, Thomas Kindler and Thomas Fischer