Mechanisms of resistance against PKC412 in resistant FLT3-ITD positive human acute myeloid leukemia cells

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Abstract Treatment of acute myeloid leukemia (AML) remains challenging with many patients harboring unfavorable prognostic parameters such as *FLT3* internal tandem duplication (*FLT3*-ITD) mutations leading to a constitutively activated FLT3-receptor tyrosine kinase (RTK). Activation of proteins by phosphorylation of tyrosine residues is a common mechanism in leukemia development. Therefore, specific tyrosine kinase inhibitors (TKI) have been developed for AML therapy and are currently under investigation. The staurosporine derivate PKC412 (Midostaurin) was found to be an effective inhibitor of the FLT3-RTK and is currently undergoing clinical trials for *FLT3*-mutated AML patients. Since resistance towards TKIs has been observed in vitro and in clinical trials, we have generated a PKC412-resistant clone (MV4-11r) of the human myelomonoblastic cell line MV4-11, which carries a homozygous *FLT3*-ITD mutation. MV4-11r displayed higher vitality after addition of PKC412 compared with MV4-11 with a pronounced reduction of apoptotic cells. Cytogenetic characterization revealed the acquisition of additional aberrations in the resistant cell line such as clonal alterations at chromosome 13q with additional *FLT3* signals. Microarray analysis revealed significant expression changes in several genes prior to and after incubation with PKC412. The expression status of candidate genes being

regulated by *FLT3*-ITD like *JAG1*, *p53*, *MCL-1*, *C-KIT*, and *FLT3-L* was confirmed by real-time PCR. In summary, resistance against PKC412 appears to be mediated by up-regulation of anti-apoptotic genes and down-regulation of proapoptotic signals as well as genes that are involved in normal and malignant hematopoiesis.

Keywords AML · Targeted therapy · PKC412 · TKI resistance · *MCL-1* · *JAG1*

Introduction

Acute myeloid leukemia (AML) is a heterogenous disease in which treatment failure affects thousands of patients each year. Evolving research activities have been able to determine risk factors in AML which help to delineate response to different individual therapeutic strategies. More recently, several novel genetic abnormalities were discovered, especially in patients with normal karyotype which allows the differentiation of AML into molecular subtypes with distinctive prognosis. Clinically unfavorable genotypes include internal tandem duplications of the *fms*-like tyrosine kinase 3 gene (*FLT3*-ITD). First described by Nakao et al. in 1996 *FLT3*-ITD harboring AML was identified as an entity which occurs in 25–35% of patients with normal karyotype [1–5]. Several studies were able to show that AML patients under the age of 60 years with *FLT3*-ITD mutations show inferior outcome [4–7]. Additionally, the allelic ratio of *FLT3*-ITD was detected to be of significance for AML patients with high mutant/wild-type ratios having a shorter overall and disease-free survival [5, 8, 9].

Since the very successful introduction of imatinib mesylate for the treatment of BCR/ABL positive chronic

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myeloid leukemia in 2001, research has focused on the development of tyrosine kinase inhibitors (TKI) for treatment of various malignant diseases [10, 11]. PKC412 (Midostaurin) initially developed as a protein kinase C inhibitor showed inhibitory activity against class III receptor tyrosine kinases (RTK) such as C-KIT, FMS, PDGFR, VEGFR 2, as well as FLT3 [12–14]. As a derivate of staurosporine, PKC412 binds to the ATP-binding pocket of FLT3 so that activation and tyrosine autophosphorylation is inhibited [15]. A phase I study indicated safety and tolerability of PKC412, respectively, whereas a phase II study on patients with progressed AML and activating mutations of *FLT3* could demonstrate a minimum of 50% blast reduction in 70% of patients in peripheral blood while six patients showed at least 50% blast reduction in the bone marrow [16, 17]. Further clinical trials for newly diagnosed *FLT3*-ITD⁺ AML patients are currently performed to further study these encouraging results. However, resistance mechanisms against TKIs were observed in leukemia cells from the very beginning including in vitro experiments and clinical research [17–20]. Thus, we were interested in identifying genes and gene patterns with unique alterations of expression levels in PKC412 sensitive or resistant *FLT3*⁺-ITD AML blasts which might contribute to the understanding of PKC412 resistance as well as prediction to PKC412 response. For these studies, we examined the sensitivity of the human AML cell line MV4-11, which carries a homozygous *FLT3*-ITD mutation, to PKC412. By generating partially resistant clones of MV4-11 in which microarray and interphase FISH studies were performed, we were able to compare both cohorts concerning cellular responses to PKC412, gene expression changes, and chromosomal alterations.

Materials and methods

Cell cultures

MV4-11 and THP-1 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ,

Braunschweig, Germany). Culturing was performed under standard conditions (37°C and 5% CO₂ in a humidified atmosphere) in 1,640 RPMI with 10% fetal calf serum (Invitrogen, Carlsbad, USA) without or with increasing dosage of PKC412. PKC412 was purchased from Calbiochem (San Diego, USA). The starting dose of PKC412 (6.6 nM) was constantly increased until a subclone of MV4-11 growing continuously with 0.13 μM PKC412 was identified (MV4-11r). The PKC412 solvent DMSO was applied as a control. Cell counting was performed using Sysmex XE-2100 (Sysmex, Kobe, Japan), and cell vitality was measured by trypan blue staining (Table 1).

MTT assay

The 3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromid proliferation assay (MTT) was performed as published [21]. Briefly, 1×10⁶ cells/100 μl/well were grown in triplicates in a 6-well plate for 48 h (with or without different concentrations of inhibitor or control). After the addition of 10 μl/well of MTT-labeling reagent and 4 h incubation, 100 μl of solubilizing agent was added. After incubation overnight, photometric measurements with a wavelength of 570 and 650 nm reference were performed.

Apoptosis assay

Samples were analyzed on a FACScanTM flow cytometer (Becton Dickinson, San Jose, USA). A minimum of 10,000 events was collected for each sample. Analysis was performed with Cellquest software (Becton Dickinson, San Jose, USA). Fluorescence activated cell sorting (FACS) analysis measurements were performed in triplicates for each cell clone. After 12 h of synchronization, cells were released from G1/S-arrest by culturing in 6-well plates in serum rich medium at a concentration of 1×10⁶/ml and addition of PKC412. For cell staining, the Annexin V-FITC (fluoresceinisothiocyanate)/PI Apoptosis Detection Kit (Alexis Biochemicals, Lausen, Switzerland) was used according to the manufacturer's instructions.

Table 1 Mean vitality parameters of MV4-11 and MV4-11r after 72 h PKC412

	DMSO		0.1μM		1μM		10μM	
	MV	MVr	MV	MVr	MV	MVr	MV	MVr
Mean vitality in % [range]	91 [87–95]	91 [91–92]	60 [51–68]	99 [99–00]	22 [15–26]	81 [80–83]	11 [4–15]	76 [74–77]
Mean apoptotic cells in % [range]	2 [1–3]	0.7 [0.6–0.8]	12 [8–17]	1 [1.1–1.3]	19 [15–25]	2 [2.3–2.5]	16 [11–25]	2 [2.1–2.3]
Mean necrotic cells in % [range]	7 [7–8]	3 [2.8–3.1]	15 [14–18]	3 [2.8–3.1]	24 [23–25]	8 [8–9]	27 [25–29]	10 [9–11]

DNA–oligonucleotide microarray

Gene expression analysis was performed in triplicate for each cell line. RNA from 45×10^6 cells was isolated for each gene expression analysis using standard methods [22]. DNA–oligonucleotide array analysis was performed as previously described [23, 24]. In brief, 10 µg of total RNA was used from each sample for the synthesis of first-strand cDNA according to the Eukaryotic Target Labeling protocol as per recommended guidelines by the manufacturer. For synthesis of hybridizable, biotin-marked cRNA [for the in vitro-transcription (IVT) reaction] the IVT protocol (Affymetrix) was performed: after purification hybridization of adjusted cRNA amounts was performed using the manufacturer's protocol [25]. Each array was scanned twice using the GeneArray scanner (Affymetrix, Carlsbad, USA). Strongest differentially regulated genes are shown in Tables 2–7 in the online supplement in order to provide lists of genes identified with gene name, accession code, mapping, and short description so that investigators may examine their gene of interest.

Real-time PCR

After isolation of RNA, reverse transcription into cDNA was performed using random hexamer priming. Real-time PCR was performed using the TaqMan Universal PCR Master Mix (containing the AmpliTaq Gold Polymerase, PCR buffer) and the Assays-on-demand Gene Expression Assay Mix (Applied Biosystems, Foster City, USA). Data analysis was performed with the Apple Macintosh Power-Mac 7200/120 and the ABI PRISM Sequence Detection Software 1.7. As control gene glyceraldehyd-3-phosphate dehydrogenase (*GAPDH*) was chosen. The Δ -Ct method was applied to describe the “fold expression change value” (fec) by calculating $2^{(\Delta - \text{Ct MVN}) - (\Delta - \text{Ct MVr})}$ [26].

Fluorescence in situ hybridization

Fifty different bacterial artificial chromosome (BAC) clones (RPCI-11 human male BAC library; BACPAC Resources, Oakland, USA) containing DNA from the chromosomal region 13q12 were selected and screened for the presence of the *FLT3* gene by PCR. BAC-DNA was isolated from clone 85P08 (Qiagen Large-Construct Kit; Qiagen, Hilden, Germany) and was labeled by nick translation (Vysis, Downers Grove, IL, USA) with fluorophore-labeled dUTP (SpectrumRed; Vysis) and used as probe for *FLT3*. FISH was performed as published before [5] using the *FLT3*-clone as well as two commercial probes [LSI (13q34) Spectrum Green and LSI 13 (RB1-13q14) Spectrum Green, both Vysis, Illinois, USA] for analysis.

Statistical analysis

In order to proof significant differences of the resistant cell line MV4-11r compared with MV4-11 the nonparametric Mann–Whitney *U* test was applied. Because analysis included measurements of both cell lines in triplicate, due to the sample size ($N_1=3$, $N_2=3$) at $U=0$ could be at least a $p=0.05$. *P* values at 0.05 were considered to be significant. *U* test calculations were performed using the SPSS software package, version 10.0 (SPSS, Chicago, USA).

In order to detect genes as present, absent, or marginal for comparative analysis of two pairs of microarrays the Affymetrix Microarray Suite program has to determine the detection *p* value. This value was determined applying the Wilcoxon test.

Results

Characterization of resistance

The vitality and fraction of apoptotic and necrotic cells of MV4-11 and the resistant cell line MV4-11r after various incubations with PKC412 is shown in Table 1. Upon incubation of MV4-11 cells in the presence of 1 µM of PKC412, a reduction of cell viability of 78% down to 22% was detected. In contrast, the resistant cell line MV4-11r showed only a reduction of vitality of 20%, with still showing 80% viable cells ($p=0.05$). Figure 1 depicts the course of viable and necrotic cells over 72 h measured by dot plot analysis in MV4-11 (a) and MV4-11r (b) after addition of 1 µM of PKC412. Annexin/PI-analysis showed an increase of apoptotic cells after incubation with 1 µM PKC412 from 3% to 19% after 24 h in MV4-11. In contrast, in MV4-11r, the increase of apoptotic cells during the same period of time was measured from 1.6% to 2.4% after 24 h ($p=0.05$). This corresponds to a reduction of the maximum apoptosis rate after 24 h compared with MV4-11 of 87%. Figure 2 shows the course of apoptotic cells in MV4-11 and MV4-11r over 72 h with 1 µM PKC412. Figure 3 depicts the course of apoptotic and necrotic cells over 72 h measured by Annexin/PI-FACS analysis in MV4-11 (a) and MV4-11r (b) after addition of 1 µM of PKC412. In parallel, an absolute cell count was performed in order to measure proliferation rates and secure correct proof of differences observed in the apoptosis assays. Figure 4a, b shows the absolute cell count measured in MV4-11 and MV4-11r over 72 h. To estimate an IC_{50} value for PKC412 for both cell lines, MTT assays with increasing dosages of PKC412 were performed. Figure 5 shows cell vitalities in MV4-11 and MV4-11r after 48 h with an estimated IC_{50} for MV4-11 of 85.6 nM compared with 341 nM in the MV4-11r clone.

Fig. 1 PKC412-induced decrease of vitality is time-dependent and decreased in MV4-11r compared with MV4-11. Flow cytometric scatterplots of **a** MV4-11 and **b** MV4-11r without PKC412 (–1), after

24 h (–2) and after 72 h (–3) of 1 μ M PKC412. Gate R1 defines necrotic cells. Gate R2 defines viable cells

Sequence analysis

Since mutations in different regulatory and drug-binding moieties of the tyrosine kinase proteins are a common mechanism of acquired resistance to targeted treatment approaches, we analyzed the entire coding region of *FLT3* in the resistant MV4-11 cells and compared it with the native cell line as well as the gene bank sequence. We could not find any additional difference in the entire coding region not present in the native MV4-11 cells.

Gene expression analysis

In order to detect gene expression changes in MV4-11 and MV4-11r which might contribute to the above-described differential sensitivity to PKC412, RNA isolation was performed after 12 h of cell cycle synchronization and 4 h after addition of 1 μ M PKC412 of MV4-11 and MV4-11r to perform DNA–oligonucleotide microarrays. All genes were compared which showed at least a two-fold (>2-fold

Fig. 2 Quantification of time-dependent PKC412-induced apoptosis of MV4-11 (MV) and MV4-11r (MVr). Apoptotic cells were labeled with Annexin V after treatment with 1 μ M PKC412 for indicated time periods and identified by flow cytometry

Fig. 3 PKC412-induced apoptosis is time-dependent and decreased in MV4-11r compared with MV4-11. Flow cytometric scatterplots of cells labeled with Annexin V and propidium iodide after indicated

time periods. **a** MV4-11 and **b** MV4-11r without PKC412 (–), after 24 h (–2) and after 72 h (–3) of 1 μ M PKC412. Gate R3 defines necrotic cells. Gate R5 defines apoptotic cells

Fig. 4 PKC412-induced decrease of cell proliferation is concentration- and time-dependent and is decreased in MV4-11r compared with MV4-11. Absolute cell counts of viable **a** MV4-11 and **b** MV4-11r after PKC412 with indicated concentrations and time points

Fig. 5 PKC412-induced decrease of vitality is concentration-dependent and is decreased in MV4-11r compared with MV4-11. Short-term viability assay (MTT, 48 h) with increasing PKC412 concentrations as indicated

up- or down-regulation) differential expression. (MV4-11 is abbreviated as “N” whereas MV4-11r is abbreviated as “R”—“0” indexes the measurement before and “4”, the measurement 4 h after application of PKC412.) First, gene expression differences being present independently before application of PKC412 were screened: 415 genes were up-regulated in MV4-11 (N0 vs. R0) and 175 genes were down-regulated in MV4-11 (R0 vs. N0). Four hours after PKC412 application differential gene expression was measured showing that 304 genes were up-regulated in MV4-11 (N4 vs. R4) and 222 genes were down-regulated in MV4-11 (R4 vs. N4). Intercluster analysis revealed that 194 genes were up-regulated in MV4-11 (N4 vs. N0), 429 genes were down-regulated in MV4-11 (N0 vs. N4), 236 genes were up-regulated in MV4-11r (R4 vs. R0), and 263 genes were down-regulated in MV4-11 (R0 vs. R4).

Several interesting, differentially up- and down-regulated genes in the microarray analysis were chosen for further analysis: The tumor-suppressor gene *p53* showed a more pronounced down-regulation at baseline in MV4-11r compared with MV4-11 (prior to treatment with PKC412) with a fold change (fc; N0 vs. R0)=56.4 (table not shown) and a down-regulation in MV4-11r compared with MV4-11 after addition of PKC412 with an fc(N4 vs. R4)=19.8, respectively. *MCL-1* was up-regulated in MV4-11r after addition of PKC412 with a fc(R4 vs. R0)=2 (Table 5). The genes for the membrane-bound ligand *JAG1* as well as its soluble form *sJAG1* were up-regulated in MV4-11r compared with MV4-11 prior to addition of PKC412 with a fc(R0 vs. N0)=20.8 for the soluble form and an fc(R0 vs. N0)=18.5 for the complete gene (tables not shown). After addition of PKC412, the up-regulation of *JAG1* with an fc(R4 vs. N4)=8.2 for the complete gene and fc(R4 vs. N4)=6.5 for the soluble form in MV4-11r compared with MV4-11 remains (Table 2). The gene for the class III RTK *C-KIT* is up-regulated in MV4-11r after treatment with PKC412 with a fc(R4 vs. R0)=3 (Table 5).

Quantitative real-time PCR

Several functionally interesting genes identified in the microarray analysis as being differentially up- and down-regulated were selected for confirming real-time PCR analysis. These candidate genes were the above-described *p53*, *MCL1*, *JAG1*, *C-KIT*, and *FLT3*. Since physiological interaction of FLT3 with its ligand FLT3-L occurs, quantitative PCR was also performed for *FLT3-L*. Figure 6 shows the fold expression change values for *p53*, *MCL1*, *JAG1*, *C-KIT*, as well as *FLT3* and *FLT3-L*. The fc of *p53* in MV4-11r (compared with MV4-11) displayed a down-regulation of 1.2 whereas, after incubation with PKC412, *p53* was up-regulated with a fc=1.6, respectively. *MCL1* was down-regulated with a fc=2.5 and up-regulated after addition of PKC412 with an fc=1.6. *JAG1* resulted having a fc=45.4 up-regulation in MV4-11r prior to PKC412 and fc=14.1 up-regulation after incubation with PKC412. *C-KIT* had an fc=1.4 prior to and fc=3.8 up-regulation after incubation with PKC412 in MV4-11r.

Cytogenetic analysis

MV4-11 is described to harbor a $t(4;11)$, +8, +19, and -21 in addition to the loss of the y chromosome. Cytogenetic investigation revealed the acquisition of several additional aberrations in the resistant cell line. Interphase FISH ($n=100-200$) with probes for *FLT3* (13q12), *RBI* (13q14), and for 13q34 revealed the disomy 13 in MV4-11 (as control) with *RBI* and 13q34 as well as *FLT3* and 13q34 in all investigated interphases. At resistance levels of 0.066 μM and 0.13 μM MV4-11r showed PKC412 subclones which are displayed in Fig. 7. Clonal alterations at chromosome 13q were observed indicating that an outgrowth of the clone showing deletion of 13q in combination with gain of additional *FLT3* signals occurs.

Fig. 6 Differential PKC412-dependent regulation of gene expression in MV4-11r. Fold change of expression of indicated genes as determined by real-time PCR after 4 h of PKC412 treatment

Fig. 7 Chromosomal aberrations in PKC412-sensitive and PKC412-resistant clones. Interphase FISH of **a** MV4-11r resistant at 0.066 μ M PKC412, **b** MV4-11r resistant at 0.13 μ M PKC412. For each stem line or subclone, $n=100$ –200 interphases were analyzed. The PKC412-sensitive MV4-11 harbored the stem line on the basis of $RB1/13q34=100\%$, $FLT3/13q34=100\%$

Discussion

The aim of this study was to compare two *FLT3*-ITD⁺ AML cell lines with different response towards the TKI PKC412. After proliferation and apoptosis assays showed a distinct resistance of MV4-11r against PKC412 by demonstrating higher proliferation rates and less apoptotic cells after addition of PKC412 as compared with the sensitive cell line MV4-11, we were interested in identifying genes with altered expression levels in both *FLT3*-ITD⁺ blasts. Therefore, we performed microarray analyses which would delineate differential gene expressions in order to detect up- and down-regulated genes in the PKC412-resistant as well in the PKC412-sensitive AML cells. Several differentially up- and down-regulated genes were chosen for quantitative PCR in order to confirm their regulation. Furthermore, we studied interphase FISH analyses to compare both cohorts of AML blasts with different PKC412 sensitivity concerning occurrence of chromosomal aberrations. Thus, the study may provide modest insight into the biology governing acquisition of TKI resistance in AML.

Imatinib mesylate was among the first selective protein kinase inhibitors developed for targeted cancer therapy [10]. While providing a prototype inhibitor, there were lessons to be learned from an emerging problem that besets

targeted therapy. For most CML patients, inhibition of the kinase activity of BCR–ABL by imatinib mesylate represents frontline therapy. But at least one fifth of patients will develop secondary resistance to the drug [27]. Among known mechanisms of BCR–ABL-dependent resistance are kinase domain mutations accounting for the vast majority of cases while others are associated with BCR–ABL overproduction through amplification or acquisition of additional Philadelphia⁺ chromosomes [28, 29]. BCR–ABL-independent resistance can be achieved among other by activation of alternative kinase signaling pathways, enhanced drug efflux from target cells or extracellular sequestration of drug molecules [30].

Although first clinical trials with TKIs such as PKC412 in AML were promising, evidence arouses that resistance towards kinase inhibitors would emerge as a clinical problem to be dealt with. Concerning PKC412, a low level of resistance was achieved in a murine, IL-3-dependent, retroviral with *FLT3*-ITD transduced Ba/F3 cell model [20]. Another in vitro screen proposed by Cools et al. identified ATP-binding pocket mutations in various Ba/F3-*FLT3*-ITD⁺ cells with varying degrees of resistance towards PKC412 [31]. Heidel et al. reported the first clinical case of an *FLT3*-ITD⁺ AML patient who acquired an ATP-binding pocket mutation under the course of PKC412 therapy

leading to relapsed disease [32]. Since DNA-sequencing of MV4-11 and MV4-11r did not reveal any mutations which would account for resistance in our model one can speculate on two previous described models conferring resistance towards PKC412 as (a) not mutated and up-regulated *FLT3*-ITD as demonstrated by Weisberg et al., (b) mutated and not up-regulated as demonstrated by Cools et al. and adding our model showing neither mutation nor up-regulation of *FLT3*-ITD. Furthermore, we wanted to know which mechanisms might contribute to the acquired resistance. Interphase FISH revealed the outgrowth of a subclone harboring a del13q with at least two and at most seven additional *FLT3* signals. Alterations involving 13q appear to be a critical step in this process. Albeit, it is well known that cytogenetic aberrations occur much more frequently in cell lines than in vivo, so that adequate comparison is difficult. On the other hand, known break-points of *FLT3* occur at 13q, emphasizing that the observed increase of additional *FLT3* signals might be specific. Surprisingly, microarray data as well as quantitative PCR did not reveal differential *FLT3* expression by means of up-regulation of the *FLT3* gene. It remains speculative why duplication of *FLT3* signals was not attended by *FLT3* overexpression. Instead differential expression of large amounts of other well known and putative genes was noted. In order to prove supposed specificity of differentially expressed genes in *FLT3*-ITD⁺ cells, we analyzed the *FLT3*-ITD⁻ AML cell line THP-1 upon PKC412 treatment

showing no changes in vitality, apoptosis, and proliferation. There was no overlap in the few genes differentially regulated at least two-fold in the THP-1 cells after treatment with PKC412 in comparison to the candidate genes found in MV4-11r after PKC412 treatment, further indicating the specificity of these expression profiles.

C-KIT was up-regulated in MV4-11r after treatment with PKC412. This class III RTK displays another RTK promoting survival, proliferation, and differentiation of hematopoietic progenitor cells with clinical significance in various malignant diseases. Although C-KIT is also inhibited by PKC412, an alternative maintenance of C-KIT activity for bypassing sufficient *FLT3*-inhibition due to differential sensitivity is speculative but among possibility [13, 33]. The highest drug efflux occurs in myeloid progenitor cells that overexpress C-KIT [34]. Interestingly, *ABC*-transporter genes were among the up- and down-regulated genes in MV4-11r and MV4-11. Among these, *ABCA1* was up-regulated after PKC412 treatment in the resistant line (R4 vs. R0) as well as the native cell line (N4 vs. N0). Down-regulated transporter genes after treatment with PKC412 in MV4-11 (N0 vs. N4) were *ABCE1*, *ABCD3*, and *ABCC1* and in MV4-11r (R0 vs. R4) *ABCC1*. *ABCC1* (also known as MRP1=multidrug-resistance-associated protein 1) was described as an efflux transporter for various drugs whereas *ABCA1*, *ABCD3*, and *ABCE1* have not been associated to specific drug efflux so far [35]. The down-regulation of the tumor-suppressor gene *p53* in MV4-11r

Fig. 8 Regulation of apoptosis under normal conditions and in the presence of PKC412. Simplified model of the interactions between MCL-1 and p53 as counterparts influencing BAK-mediated apoptosis—**a** in a non-resistant cell, **b** in resistant cell

compared with MV4-11 prior to PKC412 application and the trace of up-regulation in both cell lines after the administration of the drug was confirmed by quantitative PCR.

The myeloid cell leukemia (*MCL-1*) gene which has been described as the counterpart of *p53* in the context of mitochondrial mediated apoptosis was up-regulated due to the influence of PKC412 in MV4-11r (Table 5) [36, 37]. This was also confirmed by quantitative PCR. Moreover, a down-regulation could be observed in MV4-11r prior to PKC412 addition. Interestingly, during the preparation of this manuscript, Breitenbuecher et al. published antibody microarray data showing precisely that in PKC412 resistant AML cells *MCL-1* is up-regulated. Subsequently, by using RNA interference, they were able to prove that suppression of *MCL-1* rescued PKC412 sensitivity [38]. Since a cytochrome-C-efflux mediated apoptosis is perpetuated through interaction of *p53-BAK* complexes which then lead to a reduced interaction of *MCL-1* and *BAK*, a differential regulation of two direct counterparts of mitochondrial apoptosis is overt in this setting. Figure 8a, b attempt to illustrate these models. Increased *MCL-1* expression was shown to be related to a dismal response to chemotherapy and an inferior outcome in AML patients as well as resistance to a variety of chemotherapeutic agents [39, 40]. A balanced down-regulation of *p53* in combination with up-regulation of *MCL-1* in MV4-11r after application of PKC412 would set a surrounding in which drug-induced apoptosis could be avoided. The genes for the membrane-bound and soluble forms of *JAG1* were found to be up-regulated in MV4-11r compared with MV4-11. The influence of PKC412 resulted in a down-regulation of *JAG1* in MV4-11r (although compared with MV4-11 the gene was still overexpressed). These data were confirmed by quantitative PCR. The Notch-signaling cascade and its ligand *JAG1* are involved in maintenance and renewal of hematopoietic stem cells and have been described as deregulated in various malignant hematopoietic disorders [41–45]. Stirewalt et al. recently identified *JAG1* expression changes in leukemic blasts compared with hematopoietic cells from healthy donors with subsequent confirmation of an increased expression of *JAG1* using microarray data from 285 AML patients. With respect to cytogenetics, *JAG1* was found to have an increased expression (shown by microarray and quantitative real-time PCR) in patients with inv(16), t(15;17), and normal cytogenetics and was low in patients with t(8;21). Concerning molecular markers, *JAG1* was expressed at higher levels in AML patients with *FLT3*-ITDs than those without the mutation [46]. Since the role of *JAG1* in leukemogenesis is unclear, the role of acquired TKI resistance still needs to be investigated in detail. However, protein levels of *JAG1* (data not shown) were not regulated in these cells, which points to a more complex (and possibly compensatory) regulatory network than so far assumed.

Several microarray studies have examined expression changes in acute leukemias identifying subgroups with prognostic significance [47–49]. However, it has to be taken into account that gene expression does not represent gene function and specific intra- and intercellular interactions of proteins may alter the effects of the molecules involved. Future studies will be required to predict response to TKI-based targeted therapy as well as to accompany treatment schedules to predict evolving resistance. Genes with putative AML-specific expression changes have been chosen for further analysis while other genes were excluded. Therefore, we have provided lists of the 25 genes which showed to have the highest differential expression status in the compared analysis in an online supplement, so that other investigators may examine how their gene of interest is expressed.

In conclusion, our studies describe two cell lines differing in their response towards the TKI PKC412 by means of resistance. Characterization of those cell lines revealed alterations at chromosome 13q in combination with additional *FLT3* signals in the resistant line displaying a critical step in the acquisition of resistance. A vast majority of differentially expressed genes whose importance still has to be defined were detected. Identified genes involve apoptotic (*p53*) and anti-apoptotic (*MCL-1*) molecules as well as genes involved in normal and malignant hematopoieses (*C-KIT* and *JAG1*) revealing them as attractive candidates for future functional studies concerning TKI-based therapy.

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