



AKN-028 induces cell cycle arrest, downregulation of Myc associated genes and dose dependent reduction of tyrosine kinase activity in acute myeloid leukemia[☆]



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ABSTRACT

AKN-028 is a novel tyrosine kinase inhibitor with preclinical activity in acute myeloid leukemia (AML), presently undergoing investigation in a phase I/II study. It is a potent inhibitor of the FMS-like kinase 3 (FLT3) but shows in vitro activity in a wide range of AML samples. In the present study, we have characterized the effects of AKN-028 on AML cells in more detail. AKN-028 induced a dose-dependent G₀/G₁ arrest in AML cell line MV4-11. Treatment with AKN-028 caused significantly altered gene expression in all AML cell types tested (430 downregulated, 280 upregulated transcripts). Subsequent gene set enrichment analysis revealed enrichment of genes associated with the proto-oncogene and cell cycle regulator c-Myc among the downregulated genes in both AKN-028 and midostaurin treated cells. Kinase activity profiling in AML cell lines and primary AML samples showed that tyrosine kinase activity, but not serine/threonine kinase activity, was inhibited by AKN-028 in a dose dependent manner in all samples tested, reaching approximately the same level of kinase activity. Cells sensitive to AKN-028 showed a higher overall tyrosine kinase activity than more resistant ones, whereas serine/threonine kinase activity was similar for all primary AML samples.

In summary, AKN-028 induces cell cycle arrest in AML cells, downregulates Myc-associated genes and affect several signaling pathways. AML cells with high global tyrosine kinase activity seem to be more sensitive to the cytotoxic effect of AKN-028 in vitro.

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1. Introduction

Acute myeloid leukemia (AML) is a life-threatening neoplastic disease characterized by several acquired genetic abnormalities and with multiple signaling pathways involved in the pathogenesis. Treatment in AML is still based on cytotoxic chemotherapy, but much effort is now being put in the identification of molecular drug targets, allowing more specific therapeutic approaches in this disease. However, progress in AML treatment has been slow and the long-term survival remains poor [1,2].

We have previously reported on AKN-028; a novel tyrosine kinase inhibitor (TKI), designed as an ATP-mimetic and with potent in vitro and in vivo activity in AML and presently undergoing investigation in an international two-part multi-center phase I/II clinical study (ClinicalTrials.gov NCT01573247) [3]. AKN-028 is a potent inhibitor of the FMS-like kinase 3 (FLT3) with an IC₅₀ of 6 nM in a biochemical assay, and also an inhibitor of KIT kinase autophosphorylation when investigated in a cellular assay [3]. AKN-028 displays in vitro anti-leukemic effect resulting in cell death in tumor samples from a wide range of AML patients, irrespective of FLT3 mutation status or level of quantitative FLT3 expression. This prompted us to further investigate the mechanism of action of AKN-028 in AML.

AKN-028 displays a sequence dependent in vitro synergy with the conventional cytotoxic agents cytarabine and daunorubicin, with better antileukemic activity when the cells are exposed to standard chemotherapy simultaneously or for 24 h before adding

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AKN-028, whereas antagonism is observed when cells are pre-treated with AKN-028 [3]. The latter is in accordance with results reported for other TKIs, and may be explained by the fact that TKIs can cause cell cycle arrest [4]. This, along with our previous finding that AKN-028 in biochemical assays also inhibits CDC-like kinase 1 (CLK1) with an IC_{50} of 140 nM and the mTOR mediator ribosomal protein 6 kinase (RPS6K) with an IC_{50} of 220 nM [3], led us to explore the possible cell cycle effects of AKN-028 in more detail, especially since RPS6K is involved in both mTOR dependent cell cycle control and effective G_1 cell cycle progression [5,6].

Genome wide expression analysis using microarray technique has become an important tool in genomic research and a method widely used in mechanism of action studies of new compounds [7]. To analyze data from microarray studies, one can take advantage of gene set enrichment analysis (GSEA) that derives its strength from focusing on whole gene sets, linked by common biological features, chromosomal location or regulation. The aim of a GSEA is to determine whether members of a predefined gene set (e.g. genes linked by a specific signaling pathway) are enriched at the extremes (top/bottom) of a pre-ranked gene list [8].

AKN-028 displays a diagnose-specific in vitro efficacy in AML cell lines, which might in part be attributed to FLT3-inhibition [3]. However, the full in vitro efficacy of a compound can rely on more than direct target–compound interaction, for example inhibition downstream of immediate interaction with a tyrosine kinase receptor. The complexity of kinase signaling cascades, with their intricate interconnected circuits, makes it necessary to study compound effect on biochemical, cellular as well as organismal level [9]. Global tyrosine kinase activity profiling has previously been reported to contribute to improved understanding of signaling pathways in leukemia, thereby offering a valuable approach in identifying the mechanism of action involved, following treatments with specific drugs [10,11].

The aim of the present study was to further characterize pathways involved in the cell death induced by AKN-028 in AML cells. Therefore, we have performed studies on cell cycle effects, gene expression studies with subsequent analysis including GSEA, as well as a global tyrosine kinase activation profiling in AML cell lines and in primary AML tumor cells exposed to AKN-028.

2. Methods and materials

2.1. Reagents

AKN-028 (*N*-3-(1*H*-indol-5-yl)-5-pyridin-4-yl-pyrazine-2,3-diamine, provided by Akinion Pharmaceuticals, Stockholm, Sweden), reference multikinase inhibitor midostaurin (purchased from LC Laboratories, Woburn, MA, USA) and All-trans retinoic acid (ATRA, Sigma–Aldrich Co, St. Louis, MO, USA) were stored at -70°C as 10 mM stock solutions in dimethyl sulphoxide (DMSO, Sigma–Aldrich) and further diluted with culture medium (Sigma–Aldrich) as needed. The reference compound UCN01 (hydroxystaurosporin) used in the serine/threonine kinase assay was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

2.2. Cell culture

Tumor cells from three patients with AML (Unique Patient Number (UPN)) 1–3 were isolated by density gradient centrifugation, stored at -150°C in 10% DMSO and 90% heat-inactivated fetal calf serum (FCS, Sigma–Aldrich) and then thawed at experimental setup. The cells were maintained in RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 U/ml penicillin at 37°C in humidified air containing 5% CO_2 (all

obtained from Sigma–Aldrich). The sampling was approved by the Ethics Committee of Uppsala University (No. 21/93 and 2007/237).

AML cell lines MV4-11 (FLT3-ITD mutated) and HL60 (FLT3 wild type), obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), were kept in Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented as above. The cell lines were subcultivated twice weekly and the number of cells was determined by trypan-blue counting.

2.3. Quantitative FLT3 expression analysis

The quantitative FLT3 expression of AML cell lines MV4-11 and HL60 was determined as described previously [3]. The amount of FLT3 transcripts was expressed as a ratio of FLT3 copy number relative to 100 copies of reference gene GUSB (mean copy number FLT3/mean copy number GUSB \times 100).

2.4. Fluorometric microculture cytotoxicity assay – FMCA

The cytotoxic activity of midostaurin was evaluated by use of the fluorometric microculture cytotoxicity assay (FMCA), described in detail previously [12]. In short, this method measures hydrolysis of fluorescein diacetate to fluorescein by cells with intact plasma membranes. Primary AML samples or AML cell lines MV4-11 or HL60 were incubated with serially diluted midostaurin at 37°C . Living cell density was assessed after 72 h with cell survival presented as survival index (SI, %) defined as fluorescence in test wells in percent of control cultures with blank values subtracted. The half maximal inhibitory concentration (IC_{50}) was determined from log concentration–effect curves in Graph Pad Prism (GraphPad software Inc., CA, USA) using non-linear regression analysis.

2.5. Cell cycle analysis

Preparation of cells and analysis of cell cycle distribution was performed as described by Vindelov et al. [13]. Briefly, cells were seeded into 6 well plates and exposed to three concentrations of AKN-028, ATRA (known to induce $G_{0/1}$ cell cycle arrest [14]) or vehicle control (DMSO). After three days, cells were washed with phosphate buffered saline (PBS), treated with 0.03 mg/ml trypsin (Sigma–Aldrich) for 10 min at room temperature, followed by addition of 0.08 mg/ml RNase A (Sigma–Aldrich) and 0.5 mg/ml trypsin inhibitor (Sigma–Aldrich). After an additional incubation of 10 min at room temperature, nuclei were stained with propidium iodide (PI, 0.2 mg/ml, Calbiochem, San Diego, CA, USA) and analyzed using a fluorescence-activated cell sorting (FACS; BD LSR II flow cytometer, BD Biosciences, Franklin Lakes, NJ, USA) and FACS DiVa software (BD Biosciences).

2.6. Gene expression analysis

Microarray analysis was performed as described by Lamb et al. [15]. Briefly, MV4-11, HL60 and leukemic cells from patient UPN2, respectively, were seeded into 6 well plates at a density of 0.2 million cells/well and then grown for 24 h. The following day, cells were exposed to AKN-028 or reference compound midostaurin at a final concentration of 10 μM or vehicle only (DMSO). Cells were incubated for 6 h, washed with PBS and total RNA was prepared using RNeasy[®] Mini Kit (Qiagen AB, Sollentuna, Sweden) according to the manufacturer's instructions.

The RNA concentration was measured with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc., Palo Alto, CA, USA). For the MV4-11 samples, 2 μg of total RNA from each sample were used to prepare biotinylated fragmented cRNA according to the

GeneChip[®] Expression Analysis Technical Manual (P/N 702232 Rev2, Affymetrix Inc., Santa Clara, CA, USA). Affymetrix GeneChip[®] expression arrays (Human Genome U133 Plus 2.0 Array) were hybridized for 16 h in a 45 °C incubator, rotated at 60 rpm. According to the manufacturer's instructions, the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip[®] Scanner 3000 7G. For HL60 and the primary patient sample, 250 ng of total RNA from each sample were used to prepare biotinylated fragmented cRNA according to the GeneChip[®] 3' IVT Express Kit Manual (PN 702646 Rev1, Affymetrix Inc., Santa Clara, CA). Affymetrix GeneChip[®] expression arrays (Human Genome U133 Plus 2.0 Array) were hybridized for 16 h in a 45 °C incubator, rotated at 60 rpm. According to the GeneChip[®] Expression Wash, Stain and Scan Manual (PN 702731 Rev2, Affymetrix Inc., Santa Clara, CA) the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip[®] Scanner 3000 7G.

2.7. Microarray data analysis

Subsequent analysis of the gene expression data was carried out in the freely available statistical computing language R (<http://www.r-project.org>) using packages available from the Bioconductor project (www.bioconductor.org). Principal component analysis (PCA) was used to visualize the data; using the 'princomp' function in Matlab. The raw data was normalized using the robust multi-array average (RMA) method first suggested by Li and Wong in 2001 [16,17]. In order to search for the differentially expressed genes between the AKN-028 treated samples and the control group only these samples were normalized together and an empirical Bayes moderated *t*-test was then applied [18], using the 'limma' package [19]. To address the problem with multiple testing, the *p*-values were adjusted using the method of Benjamini and Hochberg [20].

To further characterize the actions of AKN-028, we performed a gene set enrichment analysis (GSEA) as described previously [8,21]. To establish the rank lists, average fold change was used as rank metric for AKN-028-treated cells versus control; fold change for midostaurin-treated samples versus control was used for comparison.

2.8. Tyrosine kinase and serine/threonine kinase activity profiling using PamChip[®] peptide microarrays

Tyrosine kinase activity profiles were determined using the PamChip[®] tyrosine kinase peptide microarray system (PamGene International B.V., 's-Hertogenbosch, The Netherlands), as described previously [22]. Serine/threonine kinase activity profiles were determined with the serine/threonine PamChip[®] peptide microarray system (PamGene International B.V.). The arrays contain 144 peptides of which two are prephosphorylated on the PamChip[®] serine/threonine kinase peptide arrays and one on the PamChip[®] tyrosine kinase peptide array (serving as positive controls). The tyrosine kinase microarray also contains one negative control. Cryopreserved leukemic cells were thawed and then lysed in M-PER Mammalian Extraction Buffer containing phosphatase and protease inhibitors (#78501, #78426, #78415, Thermo Scientific, Waltham, MA, USA), incubated on ice for 15 min and centrifuged at 10,000 × *g* for 15 min at 4 °C after which the supernatant was collected and stored in aliquots at –80 °C. The protein concentrations in the lysates were determined according to the manufacturer's instruction using the Micro BCA[™] Protein Assay kit (#23235, Thermo Scientific).

The incubations were performed on the PamStation[®] 12 according to the instructions in the PamGene PTK assay kit (PamGene International B.V.). In short, before addition of the samples, the PamChip[®] arrays were blocked with 2% bovine serum

albumin (BSA) according to the standard settings for the PamStation[®] 12. The reaction mixture contained 100 μM ATP, 0.5% DMSO and 2–5 μg protein lysate per array for the tyrosine kinase assay. Since the optimal amount of lysate per array varied depending on the activity of the protein kinases in the lysate with regards to signal intensity, the input concentration for the tyrosine kinase assay was optimized for each sample. AKN-028 or vehicle (DMSO) was added as a spike in to the incubations in concentrations ranging from 0 to 25 μM. The concentration range of the compound was chosen in order to obtain complete in vitro efficacy.

Peptide phosphorylation of the 144 peptides on the array was detected during the kinetic run with the FITC labeled anti-phosphotyrosine antibody PY20. Images of the arrays were taken every 5 cycles for 60 cycles. The arrays were then washed and a set of images was taken at different exposure times. Experiments were performed in triplicates or more. Serine/threonine kinase activity was measured for the three patient samples, essentially as described for the tyrosine kinase assay. Peptide phosphorylation was detected with a mixture of anti-phosphoserine/threonine antibodies and visualized with a FITC labeled secondary antibody. To account for differences in protein input, signal intensities were normalized to the array mean, 1 μg per array was used for all samples in the serine/threonine kinase assay after optimization of the sample input, 10 μM AKN-028, 10 μM UCN-01 or vehicle (DMSO) was added as a spike in to the incubations.

After visual check of correct peptide spot identification, signal intensities on each peptide at each time point were quantified using the Bionavigator software (PamGene International BV). Data analysis was performed with the same software package. Signal intensities minus the local background of the after wash images were normalized to exposure time, excluding signals on peptides where more than 5% of the pixels were saturated. For the tyrosine kinase assay, three peptides had to be excluded because saturation occurred at all exposure times. The kinetics of the tyrosine kinase reaction was used for quality control: only those peptides were included where signals increased in time in at least one sample in the absence of AKN-028. Furthermore, signal intensity had to be higher than the local background value. For analysis of the serine/threonine data, only peptides that showed ATP dependent signals were included in the analysis.

2.9. Statistical analysis

Statistical analysis of the gene expression data is described in detail in Section 2.7 and the IC₅₀ calculations in Section 2.4. For comparison between groups in the kinase activity assays, Student's *t*-test was used, *p* < 0.05 was considered significant.

3. Results

3.1. AKN-028 induces cell cycle arrest in the MV4-11 cell line

For the cell cycle distribution analysis, the MV4-11 cells were induced with AKN-028, the positive control ATRA or vehicle control only (0.1% DMSO) and cell cycle distribution analyzed after 72 h exposure. Treatment with AKN-028, as well as ATRA resulted in G₀/G₁ cell cycle arrest and increased cell death, as observed by the increased size of the pre-G₁ phase nuclei peak (Fig. 1a–c). Notably, the cell cycle effect of AKN-028 displayed a dose dependent pattern as shown in Fig. 1d.

3.2. Downregulated genes in AKN-028 treated cells are associated with the Myc pathway

The cytotoxic activity of AKN-028 in the AML cell lines MV4-11 and HL60 as well as in the three primary AML samples has

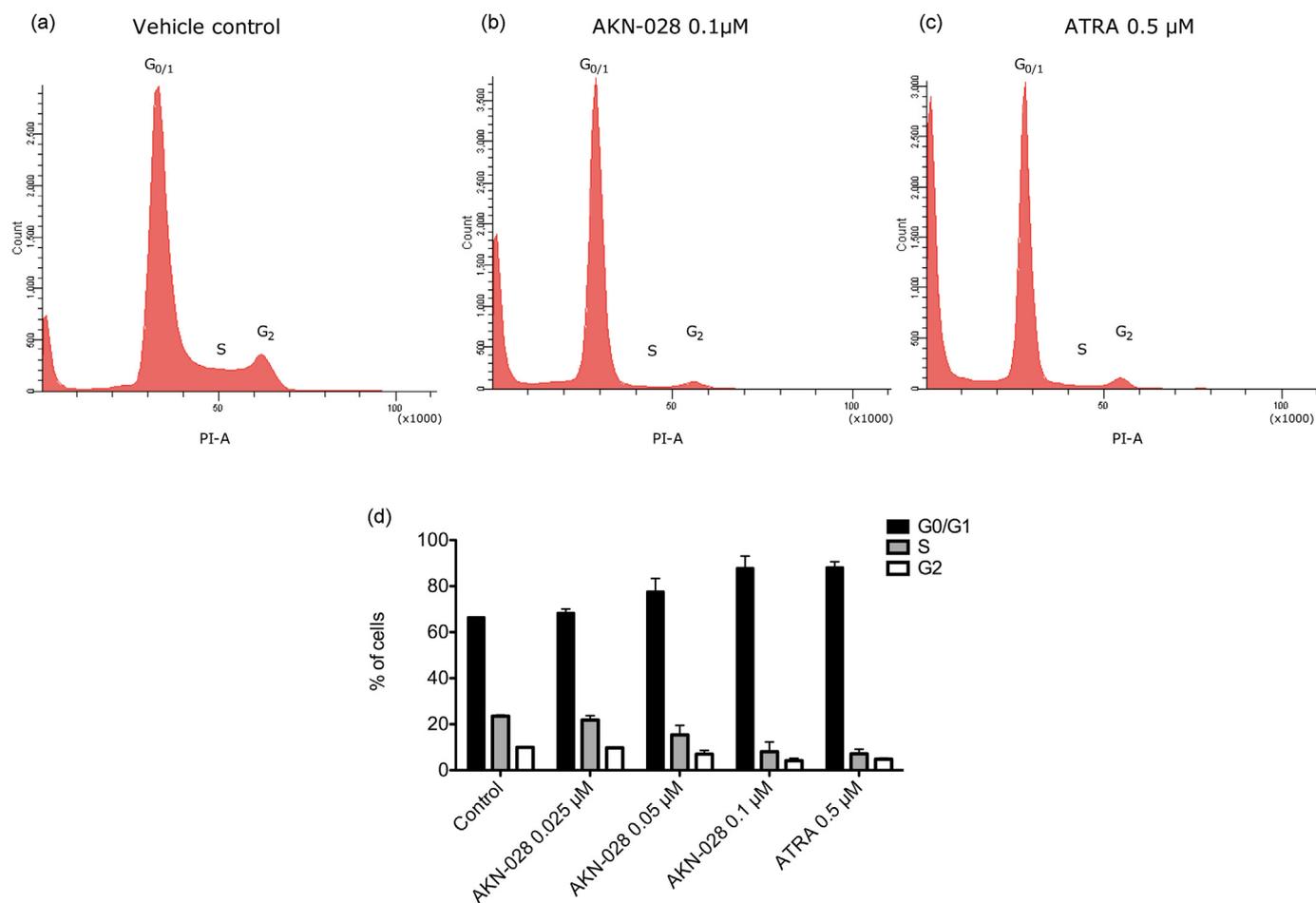


Fig. 1. Cell cycle distribution of MV4-11 cells 72 h after induction with vehicle (a), 0.1 μM AKN-028 (b) or 0.5 μM of positive control ATRA (c). $N = 4$, one typical experiment analyzed by flow cytometry displayed. (d) shows the percentage of MV4-11 cells in different phases of the cell cycle after treatment with 0.025, 0.05 or 0.1 μM of AKN-028, 0.5 μM of positive control ATRA or vehicle only (0.1% DMSO), mean values, error bars represent SEM.

previously been evaluated by the FMCA [3]. The cytotoxic activity of reference compound midostaurin was now evaluated in the cell lines and in UPN2 by use of the FMCA (IC_{50} values for MV4-11: 0.0199 μM, HL60: 20.3 μM and UPN2: 0.465 μM). Cell lines were examined regarding overall quantitative FLT3 expression, displaying a ratio $FLT3/GUSB \times 100$ of 88.52 for MV4-11 and 8.114 for HL60.

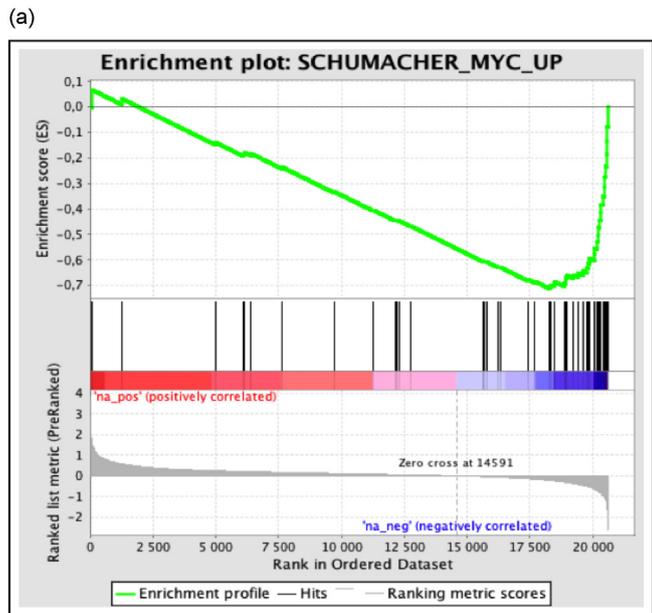
The effects of AKN-028 in AML were further investigated by measurement of changes in gene expression in compound-treated AML cell lines and primary cells from one AML patient (UPN2). The MV4-11, HL60 and UPN2 cells were exposed to 10 μM AKN-028, the reference compound midostaurin, or vehicle (DMSO) for 6 h. Treatment with AKN-028 resulted in significantly altered gene expression, with similar pattern for all samples in all three cell types tested, compared to the vehicle-treated cells. In total, 430 transcripts were downregulated and 280 were upregulated (>twofold difference, mean signal >5 and adjusted $p < 0.05$).

A gene set enrichment analysis (GSEA) of microarray results generated from AKN-028 or midostaurin treated leukemia cells and common for all samples was performed to promote better understanding of the mode of action of the compounds in AML. The analysis revealed an enrichment of predefined genes associated with the proto-oncogene c-Myc [23] among the downregulated genes in both the AKN-028 and the multikinase inhibitor midostaurin treated cells (Fig. 2).

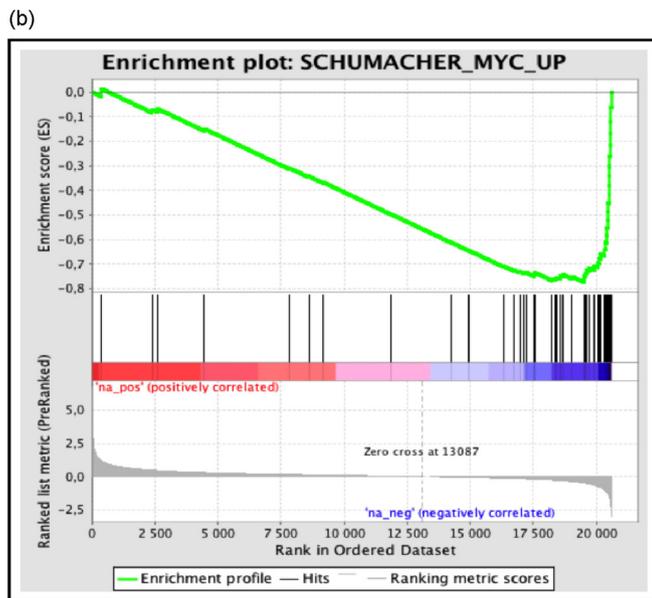
3.3. AKN-028 sensitive AML cells tend to show a higher overall tyrosine kinase activity than more resistant samples

To further investigate the actions of AKN-028, the tyrosine kinase activity profile was measured in lysates from the two AML cell lines as well as the three primary AML samples. The AML cell lines and the primary patient samples were selected from their previously known in vitro sensitivity to AKN-028 (IC_{50} values for MV4-11: 0.0283 μM, HL60: 5.87 μM, UPN1: 11.3 μM, UPN2: 0.890 μM and UPN3: 0.420 μM [3]). Altogether, the patient sample UPN1 and the cell line HL60 represent relatively resistant samples, while UPN2 is an intermediate variant and UPN3 and MV4-11 are relatively sensitive to AKN-028.

Comparison of the tyrosine kinase activity profile between the AKN-028 resistant and sensitive cell types revealed a significant difference (p -value < 0.05, Student's t -test) in basal phosphorylation level in 57 of the 141 peptide substrates tested. Both cell lines were actively proliferating at a comparable rate when the lysates were prepared. For primary AML samples, this pattern was even more pronounced with 73 out of the 141 peptides showing significantly higher peptide phosphorylation signals (p -value < 0.05) in UPN3 as compared to the more resistant UPN1. The differences in tyrosine kinase activity as read out on the peptides with the highest signal intensity are presented in Fig. 3, showing identical peptide sets for the different cell types. We found several peptides that are phosphorylated by tyrosine kinases involved in



AKN-028



Midostaurin

Fig. 2. Gene set enrichment analysis based on microarray data from leukemic cells (cell lines MV4-11, HL60 and primary AML sample UPN2) treated with 10 μ M of AKN-028 (a) or midostaurin (b) compared to vehicle control. Genes have been rank-ordered according to their relative fold change. Enrichment profiles show a downregulation of Myc-associated genes upon treatment with AKN-028 or midostaurin.

tumor cell proliferation and/or survival, such as Cdk2, Src and Src-related kinases.

3.4. Tyrosine kinase activity in AML cells is inhibited by AKN-028 in a dose dependent manner

The tyrosine kinase activity in lysates from AML cell lines and from the three primary patients was analyzed in the presence of AKN-028. In all samples tested, 10 μ M of AKN-028 reduced peptide tyrosine phosphorylation to approximately the same level (Fig. 4a and b). Since all samples reached the same level of inhibition, and bearing in mind the difference between sensitive

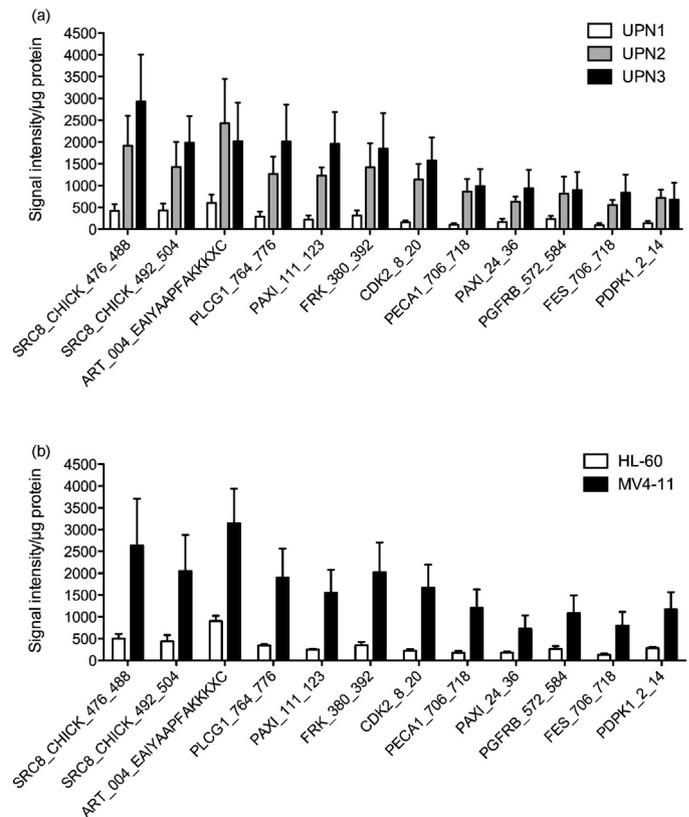


Fig. 3. The twelve peptides with the highest basal signal after phosphorylation by tyrosine kinases present in lysates of three primary AML samples (a) and two AML cell lines (b), error bars represent SD, $n = 3-6$. UPN1 and cell line HL60 represent more resistant samples, UPN2 an intermediate variant and UPN3 and cell line MV4-11 more sensitive ones with regards to cytotoxic activity of AKN-028 in vitro. Results are presented as signal intensity/ μ g protein.

and resistant samples regarding basal tyrosine kinase activity, the results indicate that the variation in sensitivity to AKN-028 in vitro might be related to the difference in overall tyrosine kinase activity between different AML cells. Using four different concentrations of AKN-028 (0–25 μ M), there was a dose-dependent inhibition (Fig. 4c) in all cell samples. As with the peptides with the highest overall tyrosine phosphorylation signal, we identified several peptides phosphorylated by kinases involved in the pathogenesis of malignancies among peptides whose phosphorylation was most inhibited by AKN-028.

Serine/threonine kinase activity of the three patient samples showed at most 30% difference in signal intensity between the samples, whereas more than five-fold differences were observed for the tyrosine kinase activity (see Figs. 4 and 5). Since such small differences could also be due to differences in input concentration, signal intensities were normalized to the mean signal per array, to eliminate such input differences (Fig. 5a). In this analysis, only 27, 16, and 15 peptides of the 142 tested were found to be significantly different between UPN2 and UPN3, UPN1 and UPN3 and UPN1 and UPN2 respectively. While 10 μ M AKN-028 led to more than 50% inhibition of tyrosine kinase activity, serine/threonine kinase activity was hardly affected (Fig. 5b). At most, 20–30% inhibition was seen in one sample (UPN2), which indicates that AKN-028 does not have strong overall inhibitory action on serine/threonine kinases.

4. Discussion

We have shown that the tyrosine kinase inhibitor (TKI) AKN-028 induces a dose-dependent accumulation of cells in the $G_{0/1}$

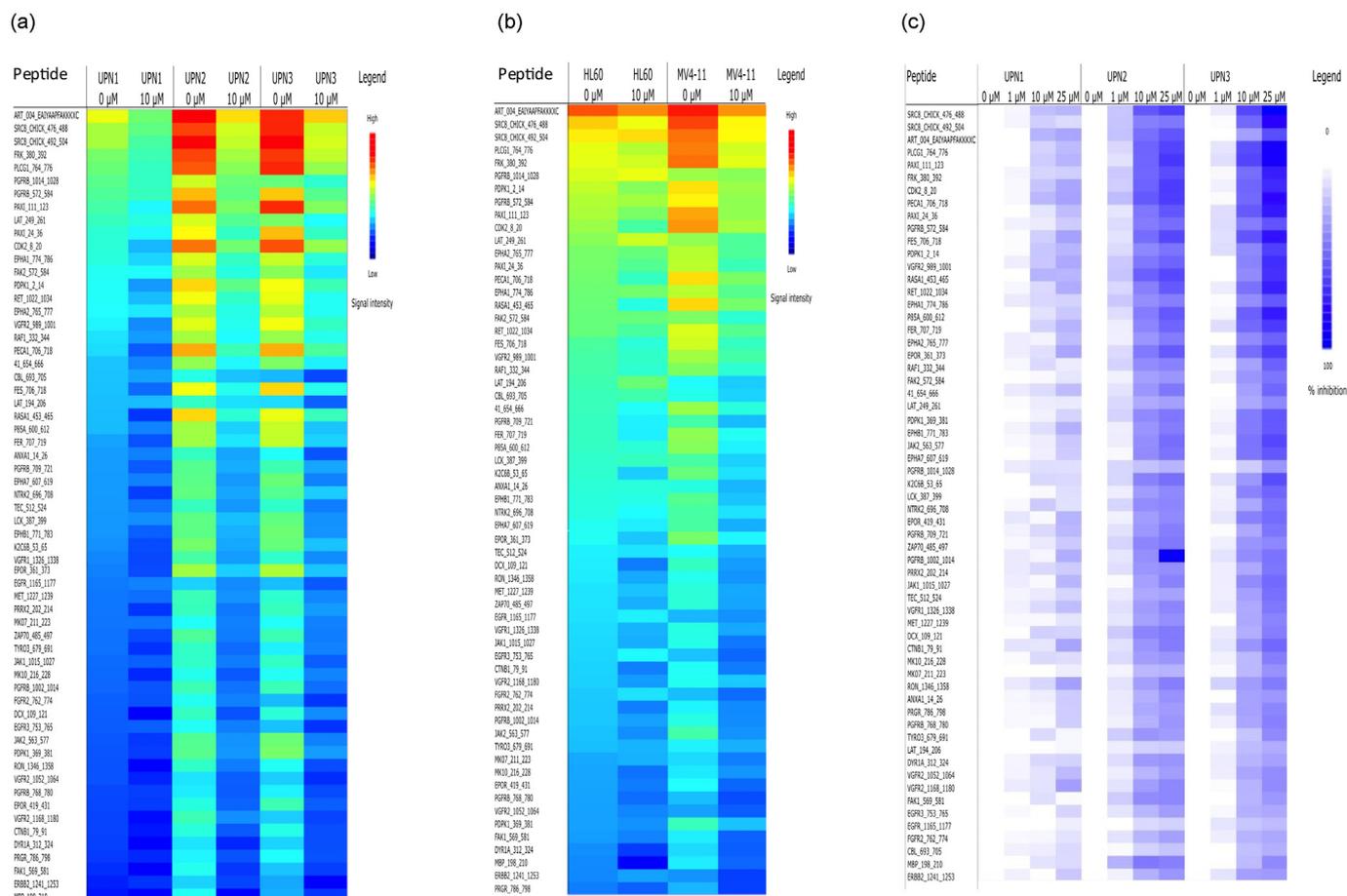


Fig. 4. Tyrosine kinase activity profiles from three AML primary patient samples (a) and HL60 and MV4-11 cell lines (b) with 10 μM of AKN-028 or vehicle (DMSO) only present during the assay. The peptide selection was made as described in Section 2. (c) shows the effect of the presence of increasing concentrations of AKN-028 in the assay on tyrosine kinase activity in AML patient samples. Signal intensity expressed as a ratio between lysate without and with the kinase inhibitor AKN-028, results presented as % inhibition.

phase of the cell cycle. This is in line with results reported for other TKIs [4,24] and compatible with a downregulation of both cyclin D-dependent kinases (Cdk) and Myc. The cell cycle arrest also offers a possible explanation for the sequence-dependent in vitro synergy previously reported when AKN-028 is combined with cytarabine or daunorubicin, i.e. the standard cytotoxic agents used in AML therapy [3].

Using a gene set enrichment analysis of microarray results generated from AKN-028 or midostaurin treated leukemia cells, we observed an enrichment of the Myc-pathway among the downregulated genes for both compounds. The proto-oncogene c-Myc encodes the transcription factor Myc, a significant regulator in hematopoiesis, which plays an important role in the control of cell proliferation, differentiation and apoptosis. Myc dysregulation is common in many types of malignant diseases including AML [23,25]. Indeed, the fusion proteins AML1-ETO, PML/RAR α and PLZF/RAR α , all associated with AML, along with activating mutations in FLT3, have been reported to cause upregulation of Myc [26,27]. Recently, bromodomain-containing 4 (Brd4) has been identified as a therapeutic target in AML. The suppression of Brd4 leads to cell cycle arrest and apoptosis, an effect, at least partly, due to the global reduction in expression of Myc target genes [28]. We know from previous studies that AKN-028 will induce apoptosis within 12–48 h and that <25% of MV4-11 cells will be alive after 72 h at the concentration used in the cell cycle experiments (0.1 μM) [3]. We thus hypothesize that the reduction in expression of Myc target genes after AKN-028-treatment, may be the cause of the observed

cell cycle G₁ arrest and subsequent apoptosis in MV4-11 cells, but the actual sequence of events remains to be explored.

Searching for differentially expressed genes, our analysis showed that treatment with AKN-028 resulted in significantly altered gene expression in all three cell samples tested, compared to vehicle control treated cells. Although the pattern for the downregulated genes after AKN-028 treatment seemed to have similarities with the gene expression pattern of midostaurin-treated cells, the pattern for the upregulated genes appeared to be unique, affecting genes involved in cellular replication processes. The impact of this is not fully elucidated, further studies are ongoing.

We have previously shown that AKN-028 does not solely exercise its effect through FLT3 inhibition, but also gives a potent KIT inhibition in a cellular assay. Furthermore, AKN-028 inhibits CLK kinases and RPS6K when tested in on-target biochemical and cellular assays [3]. Since direct target–compound interaction not necessarily gives the full explanation of the complete effect on signaling pathways of a compound; a global kinase activity profiling technique [22] was used in this study to further understand the cellular effects of AKN-028 in AML samples. In this current study, instead of measuring phosphorylation levels of specific proteins, we wanted to measure kinase activity directly and the modulation thereof by AKN-028, using the peptides on the chip as a readout. As several peptides represent the activity of a single kinase, the effect of a compound can also be detected by multiple peptides. In addition, single peptides on the chip can be recognized by more than one tyrosine kinase due to the

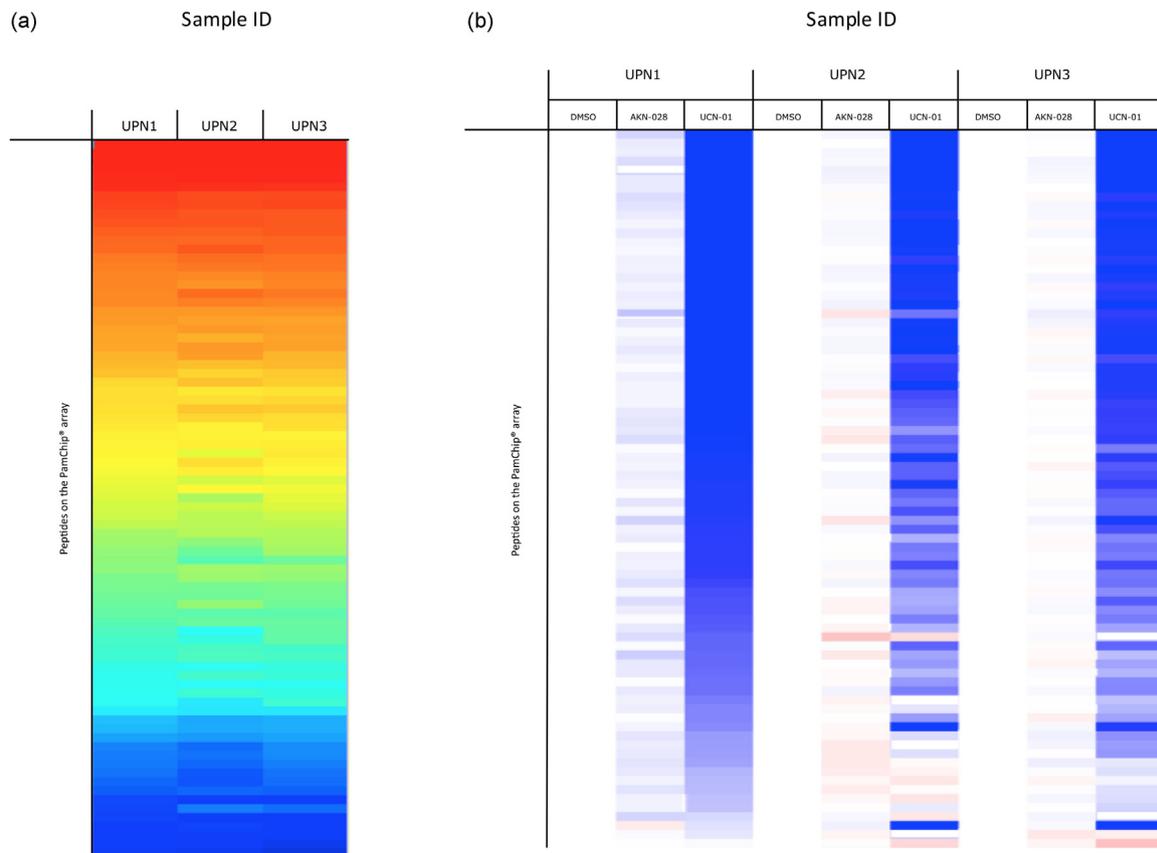


Fig. 5. Difference in basal phosphorylation activities after normalization to the array mean between three AML primary patient samples in the serine/threonine kinase assay (a) and after the addition of vehicle (DMSO), 10 μ M of AKN-028 or the positive control UCN01 (b). Signal intensity in (b) is expressed as a ratio between lysate without and with compound addition, results presented as % inhibition.

promiscuous substrate recognition of kinases. Finally, kinases as FLT3 are part of signaling networks with multiple feedback mechanisms. A kinomic approach enables measurement of overall kinase activity of a signaling network executed by a relatively specific inhibitor as AKN-028 in an AML cell. Large differences in tyrosine kinase activity were observed between the sensitive and resistant samples, whereas differences in serine/threonine kinase activity were small. Among the peptides with the highest overall tyrosine phosphorylation signal, as well as those whose phosphorylation was most inhibited by AKN-028, we identified several peptides phosphorylated by kinases involved in the pathogenesis of malignancies, including Src and Src-related kinases such as FER, FYN and FYN-related kinase (FRK). These kinases are important mediators of tumor cell proliferation and survival, and act, at least partly, through extensive interaction with receptor tyrosine kinases. Src activation also stabilizes focal adhesion complexes such as paxillin and focal adhesion kinase (FAK) [29]. Indeed, recent studies by Leischner et al. identify Src as a signaling mediator in FLT3-ITD AML, leading to activation of STAT5 [30]. Furthermore kinases FER and FES and PLCG1 are linked to FLT3 activity, as well as activation of MAPK and AKT/mTOR pathways [31,32].

In addition, the Cdk2 peptide sequence was among the top peptides whose activity was inhibited by AKN-028. Cdks are a family of kinases, forming heterodimeric complexes with a cyclin partner to become active. Cdk 2, 4 and 6 have important roles in cell cycle management as regulators of the transition between G₁/S, S-phase progression and G₂/M transition [33,34]. Moreover, downregulation of Myc genes has been shown to lead to a reduction in Cdk2 kinase activity, compatible with our observed results [34]. Interestingly, Myc signaling is induced by growth

factors, including FLT3, and mediated by cellular Src, thus providing a possible mechanism for the Myc downregulation [26,35]. As previously shown, AKN-028 induces an inhibition of FLT3 within minutes [3] and we now show a reduced phosphorylation of peptides phosphorylated by kinases such as Src and FRK within hours. Downregulation of Myc-associated genes was detected after 6 h, thereby preceding both apoptosis [3] and cell cycle effects in AML cells. In summary, we hypothesize that although AKN-028 is not a direct inhibitor of Src in a biochemical assay (data not shown), inhibition of FLT3 might reduce the activity of Src and Src related kinases, in turn causing reduced activity in the Myc-pathway, leading to subsequent cell cycle arrest and/or apoptosis. Evidently, this hypothesis needs to be further investigated, including studies of specific inhibition of Src activity in a cellular assay are warranted.

Results from the tyrosine kinase activity profiling suggest that AML samples sensitive to AKN-028 have a higher overall kinase activity than the more resistant ones. These findings imply a variance in biology between different AML patients, not necessarily linked to known mutations or karyotypic alterations. Nevertheless – all samples showed a dose dependent inhibition to AKN-028 and reached approximately the same level of activity after inhibition. The results suggest that the difference in cytotoxic activity of AKN-028 may be due to the variation in basal overall tyrosine kinase activity between cells. Obviously, larger sets of patient samples are needed before making wider generalizations, but one may speculate that tyrosine kinase activity profiling could, in the future, become an additional way of classifying AML cases in order to predict good responders, i.e. serve as a predictive biomarker.

In summary, we investigated the mode of action of the FLT3 and KIT inhibitor AKN-028 in AML, showing that it induces a G_{0/1} cell

cycle arrest. Gene expression profiling experiments revealed a downregulation of Myc-associated genes. Global tyrosine kinase profiling showed that AKN-028 affects several kinases/signaling pathways, including Cdk2, Src and Src-related kinases, which are important for tumor cell proliferation and survival. AML cells with high global tyrosine kinase activity were more sensitive to the cytotoxic effect of AKN-028 *in vitro*.

Conflict of interest

A. Eriksson has received an unrestricted research grant from Akinion Pharmaceuticals AB and is a co-investigator of the ongoing clinical trial. V. Parrow is an employee and part owner of Akinion Pharmaceuticals AB. M. Höglund is Principal Investigator for the ongoing international Phase 1–2 trial with AKN-028 in advanced AML. R. Hilhorst, R. de Wijn and L. Hovestad are employees of PamGene International B.V.

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