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Identification of AKN-032, a novel 2-aminopyrazine tyrosine kinase inhibitor, with significant preclinical activity in acute myeloid leukemia

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ABSTRACT

Aberrant signal transduction by mutant or overexpressed protein kinases has emerged as a promising target for treatment of acute myeloid leukemia (AML). We here present a novel low molecular weight kinase inhibitor, AKN-032, targeting the FMS-like tyrosine kinase 3 (FLT3) and discovered in a new type of screening funnel combining the target therapy approach with sequential cellular screens. AKN-032 was identified among 150 selected hits from three different high throughput kinase screens. Further characterization showed inhibitory activity on FLT3 enzyme with an IC_{50} of 70 nM. Western blot analysis revealed reduced autophosphorylation of the FLT3-receptor in AML cell line MV4-11 cells after exposure to AKN-032. Flow cytometry disclosed cytotoxic activity against MV4-11, but not against non-malignant 3T3-L1 fibroblast cells. Using a fluorometric microculture cytotoxicity assay, AKN-032 was tested against 15 cell lines and displayed a potent cytotoxic activity in AML cell lines MV4-11 (IC_{50} = 0.4 μM) and Kasumi-1 (IC_{50} = 2.3 μM). AKN-032 was also highly cytotoxic in tumor cells from AML patients in vitro. Furthermore, AKN-032 demonstrated significant antileukemic effect in a relatively resistant in vivo hollow fiber mouse model. No major toxicity was observed in the animals. In conclusion, AKN-032 is a promising new kinase inhibitor with significant in vivo and in vitro activity in AML. Results from the hollow fiber mouse assay suggest a favorable toxicity profile. Future studies will focus on pharmacokinetic properties, toxicity as well as further clarifying the mechanisms of action of AKN-032 in AML.

1. Introduction

Target based drug discovery has been widely used in the development of new therapies. The idea is to find potent and selective compounds, thus avoiding unwanted off-target effects [1]. The approach has been successful in developing new drugs for cancer treatment, with imatinib mesylate against chronic myeloid leukemia (CML) being one of the most notable examples [2]. The success of this compound as antileukemic treatment is based on the inhibition of Bcr/Abl, a mutated tyrosine kinase that is expressed in the CML tumor cells and upon which the CML cell proliferation depends [3]. Although targeted cancer therapy is an appealing approach, the clinical progress has been uneven and sometimes limited to a subset of patients. This has for instance been due to differences in the mutational status of oncogenes among patients or the acquisition of drug resistance [4]. Thus, an optimal kinase inhibitor may be one that combines targeted inhibition with an effect on other kinases involved in proliferation and/or apoptosis [4,5].

Acute myeloid leukemia (AML) is a heterogeneous group of diseases distinguished by uncontrolled proliferation and maturation arrest of early clonal myeloid cells in the bone marrow, leading to decreased production of normal hematopoetic cells. Despite intense chemotherapy, sometimes including allogenic stem cell transplantation, the majority of patients ultimately succumb to their disease [6]. There is an unmet need for new therapies to improve the outcome for AML patients. Thus, several new and

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more specific targeted antileukemic agents have been developed and are presently undergoing evaluation in clinical trials [7,8].

The FMS-like tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase (RTK) expressed on early hematopoetic progenitor cells, expression normally being lost upon differentiation. FLT3 is also present at high levels on tumor cells in the majority of acute leukemias [9]. Upon interaction with its ligand, the FLT3-tyrosine kinase domain is activated, causing enhancement of cell proliferation and reduction of apoptosis. Activation of FLT3 affects downstream targets in the AKT pathway, STAT and MAPkinases [10]. About 20–30% of all AML patients display activating mutations in FLT3, resulting in constitutive activation of the receptor in absence of the ligand. Some of the FLT3-mutations are associated with increased relapse rate and reduced overall survival [11,12]. Several FLT3-inhibitors have been developed, some are presently being studied in clinical trials in AML [10]. However, used as single agents, the clinical response of hitherto tested putative FLT3-inhibitors has in general been limited [13,14], although recently published case reports suggest that occasional patients may be good responders [15].

In this study, we have used a screening funnel, combining a target based approach with sequential cellular screens, in order to find the optimal compound showing efficacy in both FLT3 wild-type and FLT3 mutated AML, without targeting unrelated cells. With this approach, we identified AKN-032: a promising new antileukemic agent meeting these criteria and suitable for further development.

2. Materials and methods

2.1. Compounds

Three different high throughput kinase inhibition screens of the Biovitrum's Compound library, directed against serine and threonine kinases, resulted in the identification of several chemical scaffolds with FLT3-inhibitory activity as an off-target effect. A subset of 150 molecules from the compound library was then selected as starting points in order to analyze FLT3-inhibitory activity. The compounds were designed to bind to the ATP-binding pocket. In the current study, we present one of these compounds, AKN-032 (for molecular structure, see Fig. 1a). For comparison we used the multi-targeted kinase inhibitor sunitinib [16] and AB200434 which displays a more FLT3 selective profile, mainly targeting FLT3, C-KIT and PDGF-R [17] (both provided by Biovitrum Compound Collection, Stockholm, Sweden. For molecular structures see Fig. 1b and c). The compounds were stored at −20 or −70?C

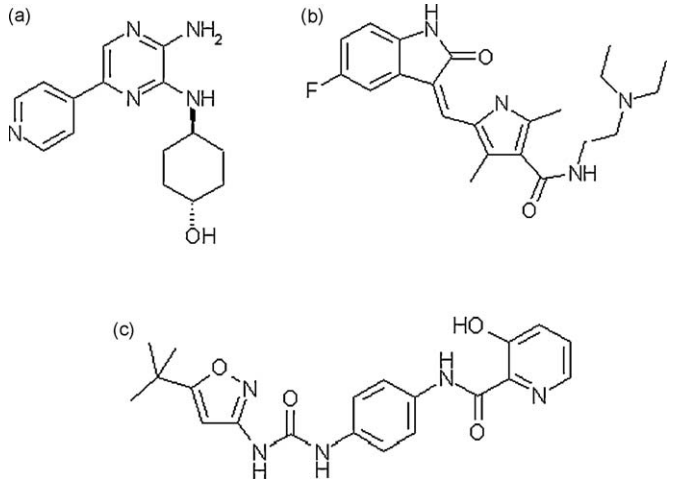


Fig. 1. Molecular structures of AKN-032 (a), sunititnib (b) and AB200434 (c).

dissolved as a 10 mM stock in DMSO and diluted with PBS or culture medium (Sigma–Aldrich Co, Stockholm, Sweden) as needed. For further comparison regarding cytotoxic activity, the conventional AML drug cytarabine (Apoteket AB, Sweden) was used.

2.2. FLT3 enzyme assay

An enzyme inhibition assay for the tyrosine kinase domain of FLT3 was established using a fluorescence polarization technique, Immobilized Metal Ion Affinity-Based Fluorescence Polarization (IMAP) from Molecular Devices (CA, USA). Kinase activity was measured by incubation of a fluorescent peptide substrate with the kinase domain (IMAP FP Exporer Progressive Binding System, Molecular Devices, #R8124, prepared according to the manufacturer's instructions). Recombinant human FLT3 enzyme was purchased from Upstate Cell Signalling Solutions (CA, USA) (flt-3 catalogue #14-500) and the fluorescently labeled (5-FAM (5-carboxyfluorescein)) substrate peptide with peptide-sequence 5FAM-KKKKEEIYFFFG-NH2 was obtained from Molecular Devices (#R7269). Final concentrations were 0.0125 U/ml for FLT3 enzyme, 100 nM for FAM-CSKtide and 100 μM for ATP. Compounds were tested in dose–response fashion, using an 11-point dilution range with 1:3 dilution steps, concentration ranging from 5000 to 0.085 nM or from 500 to 0.008 nM. Assay incubation time was 2 h. Fluorescence was measured using a plate reader (Analyst AD, Molecular Devices) with excitation wavelength 485 nm and emission wavelength 530 nm, integration time of 0.1 s.

2.3. Kinase inhibition panel screen and broad panel safety assessment

A 10 mM stock solution of AKN-032 was sent to Upstate Laboratories (NY, USA) for evaluation of kinase inhibitory activity over a panel of 31 kinases at 10 μM using homogeneous time resolved fluorescence assays (HTRF®) [18] according to Upstate standards. AKN-032 was also tested against a MDS Pharma broad selectivity safety assessment panel consisting of 71 different radioligand binding assays for receptors, ion channels and transporters (MDS Pharma Services, Taipei, Taiwan) at 1 and 10 μM according to MDS Pharma standards.

2.4. AKT and ERK protein kinase assay

A radiometric protein kinase assay, using FlashPlatesTM from Perkin Elmer (Boston, MA, USA) in a 50 μl reaction volume according the protocol of ProQinase, (33PanQinase® Activity Assay, ProQinase GmbH, Freiburg, Germany) was used for measuring the kinase activity of AKT1, 2, 3 and ERK1, 2. Grade of inhibition of kinase activity (%) was determined after 60 min incubation with 10 and 1 μM of AKN-032. Staurosporine (kindly supplied by ProQinase) was used as a reference compound.

2.5. Cell culture

AKN-032 was tested against a panel consisting of 15 different cell lines. The panel included the myeloma cell line RPMI 8226/S and its sublines 8226/Dox40 and 8226/LR5 (kind gifts from WS Dalton, Department of Medicine, Arizona Cancer Center, University of Arizona, Tucson, AZ, USA), the lymphoma cell line U-937 GTB and subline U-937-vcr [19] (kind gifts from professor K. Nilsson, Rudbeck Laboratory, Uppsala, Sweden), the acute lymphocytic leukemia cell line CCFR-CEM and subline CEM/VM-1 (kind gifts from WT Beck, Department of Pharmacology, College of Medicine, University of Tennessee, Memphis, TN, USA), human retinal pigment epithelial cell line hTERT-RPE1 (Clonetech Laboratories, Palo Alto, CA, USA), the small-cell lung carcinoma cell line NCI-H69

and its subline H69AR, the renal adenocarcinoma cell line ACHN, the cervix adenocarcinoma cell line HeLa, and 3 AML cell lines: MV4-11 (expressing a naturally occurring 30 bp FLT3/ITD mutation in homozygous form) [20,21], Kasumi-1 (harboring a *t*(8:21) mutation) [22] and HL-60 (reported to be FLT3 negative and with ability to differentiate spontaneously) [23–25] (all obtained from American Type Culture collection; ATCC, Rockville, MD, USA). A table summarizing the origin and special features of the cell lines is available as supplemental material. All cell lines except HeLa, hTERT-RPE1, MV4-11 and HL-60 were grown in culture medium RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin at 37 °C in humidified air containing 5% CO_2. HeLa was grown in Minimum Essential Medium Eagle with 1 mM sodium-pyruvate, hTERT-RPE1 in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (1:1) and MV4-11 and HL-60 in Dulbecco's Modified Eagle's Medium, all supplemented as above. All cell culture reagents were obtained from Sigma–Aldrich Co. Mouse embryonal fibroblasts 3T3-L1 (ATCC) were used between passages 4-12, cells were subcultivated twice weekly in Dulbecco's Modified Eagle's Medium supplemented with 10% FCS.

2.6. Patient samples

A total of 13 cell samples obtained from bone marrow or peripheral blood of patients with newly diagnosed or relapsed adult AML were used. Tumor cells were isolated by density gradient centrifugation, cryopreserved in 10% DMSO and 90% inactivated calf serum by initial freezing for 24 h at −70 °C, followed by storage at −150 °C and then thawed at experimental setup. Cell culture medium RPMI 1640 (Sigma–Aldrich Co), supplemented as described above, was used throughout. Cell viability was determined by Trypan-blue exclusion test and the proportion of tumor cells in each cell preparation assessed by inspection of May–Grünwald–Giemsa stained cytocentrifuge preparations [26]. The sampling was approved by the Ethics Committee of Uppsala University.

AML cell samples were examined regarding FLT3-mutation status. For FLT3-internal tandem duplications (FLT3-ITD), genomic DNA was analyzed by PCR followed by capillary electrophoresis on an AB3130XL genetic analyzer using GeneMapper™ software (Life Technologies, Foster City, CA, USA) for fragment analysis. For FLT3-tyrosine kinase domain point mutations in codon D835 (FLT3-TKD), genomic DNA was analyzed by PCR as described previously [12]. Clinical characteristics, FLT3-mutation status and karyotype of the patients are summarized in Table 1.

2.7. Western blot

MV4-11 cells were incubated for 15 h with one of six concentrations of AKN-032. Protein was extracted from cells using RIPA buffer supplemented with phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and protease inhibitor cocktail (Santa Cruz Biotech, CA, USA). Protein concentrations were measured by bicinchoninic acid protein assay [27] and 1000 μg protein from each sample was incubated with anti-FLT3-antibody (#sc-20733, Santa Cruz Biotech), separated by NuPAGE (Invitrogen Ltd, Paisley, UK #LC5602), transferred to PVDF-membranes (Bio-Rad Laboratories, Sundbyberg, Sweden) and probed with primary antibody (p-Tyr, #sc7020, Santa Cruz). A secondary antibody (conjugated with horseradish peroxidase (HRP)) was used for chemiluminiscense detection (Amersham ECL Plus detection kit, GE healthcare, Uppsala, Sweden). The membrane was stripped of antibodies and reprobed using a goat antibody against FLT3 (#F0550, Sigma–Aldrich Co) and a HRP-conjugated rabbit anti-goat secondary antibody (#P0449, Dako Sweden AB, Stockholm, Sweden).

2.8. The fluorometric microculture cytotoxicity assay

The fluorometric microculture cytotoxicity assay (FMCA), described in detail previously [28], is a method based on the measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes. FDA (Sigma–Aldrich Co) was dissolved in DMSO and kept frozen (−20 °C) as a stock solution (10 mg/mL) protected from light.

For screening of cytotoxic effect, dose–response plates containing compounds were prepared as described previously [29]. In short, the compounds were transferred to 384-well microtitre plates in duplicate wells in 10- or 5-fold serial dilution, starting from 100, 25 or 10 μM. A 1% DMSO control served as vehicle control. The plates were stored at −70 °C until further use. Cells were then seeded into the drug-prepared 384-well plates at varying density per well depending on cell type (AML: 25,000 cells/well, NCI-H69 and H69AR: 2500 cells/well and all other cell lines: 5000 cells/well). Plates were incubated at 37 °C for 72 h, and cell density measured using the FMCA.

For accurate IC_{50} determination, 3000–5000 MV4-11 cells/well were seeded in 50 μl culture medium supplemented with 10 or 20% heat-inactivated fetal calf serum into a 96-well plate. Threefold serial dilution from a DMSO stock of AKN-032, sunitinib or AB200434 was made in serum-free culture medium supplemented with penicillin and streptomycin and then added to the

Table 1
Acute myeloid leukemia patients (*n* = 13). Clinical characteristics, FLT3-mutation status and karyotype. FAB indicates French–American–British classification; n.d. – not done; complex karyotype indicates karyotype with three or more aberrations.

Sample (no)	Age (years)	Sex	Subclass (FAB)	Status	FLT3 ITD	FLT3 D835	Karyotype
1	53	M	M2	Relapse	wt	wt	Complex
2	38	M	n.d	Relapse after allo SCT	wt	wt	Complex
3	28	F	M2	Relapse	wt	mut	46, XX
4	55	F	M4	Newly diagnosed	wt	wt	46, XX, (45, XX, −9)
5	61	M	M2	Relapse	mut	wt	46, XY
6	22	F	M1	Newly diagnosed	wt	wt	Failed, 46XX after SCT
7	58	M	n.d	Relapse	wt	mut	n.d.
8	53	M	M0	Relapse	wt	wt	Complex
9	68	M	M1	Newly diagnosed	mut	wt	46, XY
10	79	M	M2	Relapse	mut	wt	46, XY
11	58	F	M2	Relapse	mut	wt	46, XX
12*	53	M	M0	Relapse	wt	wt	Complex
13*	47	M	M1	Newly diagnosed	wt	wt	46XY,inv(16)(p13q22)(25)

* Used in the hollow fiber study.

cells, 50 μl of the serial dilutions were added to the cell-suspension. The final concentration ranged from 5 μM to 0.8 nM, or from 500 nM to 0.08 nM, all wells containing 0.5% DMSO. A 0.5% DMSO control served as vehicle control. For comparison, the conventional AML drug cytarabine (Apoteket AB, Sweden) was serially diluted as described above, final concentrations ranging from 25 μM to 4 nM. The cells were incubated for 72 h at 37 °C and the total number of viable cells then measured by use of the FMCA.

For the FMCA, cell survival after exposure to cytotoxic drugs is presented as survival index (SI, %) defined as fluorescence in test wells in percent of control cultures (cells in culture medium), blank values subtracted. FMCA was considered successful only when fluorescence signal in control cultures was >5× of mean blank values, a mean coefficient of variation in control cultures was < 30% and tumor cell fraction surpassed 70% after incubation.

2.9. Flow cytometry analysis

The effect of compounds on viability and proliferation was also analyzed using flow cytometry. MV4-11 or 3T3-L1 cells were seeded, treated with compounds in serial dilution and incubated as described for the accurate IC_{50} determination. After 72 h, 100 μl viability reagent (Guava ViaCount, staining dead cells) diluted in PBS according to manufacturer's instruction, was added to each well in the drug-prepared 96-well plates, resulting in a total volume of 200 μl. The 3T3-L1 cells were trypsinised and resuspended in 100 μl serum-free medium prior to addition of viability reagent. Number of viable cells was determined using Guava 96-well ViaCount assay (Guava Technologies, CA, USA) according to manufacturer's instructions. Survival (%) was calculated as number of viable cells per ml compared to vehicle treated cells (0.5% DMSO) at the end of experiment.

2.10. Apoptosis assay

A high content screening assay for measurement of apoptosis was used to examine death characteristics of cells exposed to AKN-032, the method has been described in detail previously [30,31]. Briefly, 12,000 cells/well of AML cell lines MV4-11 and HL-60 were plated in flat-bottom 96-well plates and exposed for 24 h to 4 concentrations of AKN-032 in duplicate wells in tenfold serial dilution starting from 50 μM. A known apoptosis-inducing agent, etoposide (Apoteket AB), was used as a positive control at a concentration of 1 or 10 μM. The FLICA probe FAM-DEVD-FMK (part of the CaspaTag apoptosis kit, Millipore, Solna, Sweden), which stains cells with activated caspases, was added 1 h before the end of drug exposure at a final concentration of 20 μM. Plates were washed in PBS, followed by fixation with 3.7% formaldehyde and 10 μM Hoechst 33342 (Sigma–Aldrich Co) for 15 min and then washed twice again. Caspase activity was analyzed by use of the ArrayScan™ high content screening system (Cellomics Inc., Pittsburgh, PS, USA), described in detail previously [30], at least 800 cells were analyzed per well.

2.11. Pharmacokinetic studies

A solution of 3 mg/ml AKN-032, 10% hydroxypropyl-beta-cyclodextrin, 20 mM sodium acetate and 0.9% sodium chloride was administered subcutaneously at a dose of 15 mg/kg to nine male C57 black mice (Scanbur, Sollentuna, Sweden). Blood samples were collected at 0.08, 0.25, 0.5, 1, 2, 4, 8, 16 and 24 h following injection (n = 3 per time point, each mouse was sampled thrice) and plasma was prepared by centrifugation (~4000 rpm, 5 min). Plasma concentrations of AKN-032 were determined by protein precipitation and electrospray LCMS. Lower limit of quantification (LLOQ) was 0.003 μM. Pharmacokinetic parameters were calculated based on median plasma concentration versus time data using WinNonlin® software version 4.1 (Pharsight Corp., USA). The area under the plasma concentration versus time curve (AUC) was calculated by the linear trapezoidal method and the plasma half-life ($t_{1/2}$) was estimated from the slope of the curve between 0.25 and 4 h. Animals were given food and water ad libitum throughout the experiment. The animals were euthanized by terminal bleeding from vena cava, under sedation with isoflurane. The study was approved by the Animal Ethics Committee in Northern Stockholm (no. N94/04).

2.12. Hollow fiber

Cells from two AML patients (see Table 1) and from the AML cell line MV4-11 were cultured inside semi-permeable polyvinylidene fluoride fibers [32]. The fibers were sealed and incubated for two days before implantation subcutaneously into NMRI male mice (Scanbur). The day following implantation, subcutaneous twice daily administration of either 20 or 40 mg/kg of AKN-032, or vehicle only (n = 8 animals/group) was started. Body weight was determined daily in connection with the first drug administration. Drug administration lasted for six days, whereupon the fibers were retrieved and cell density evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)-assay [33]. This method is based on the conversion of MTT to blue formazan crystals by living cells. The formazan was extracted by DMSO as previously described [34], and optical density (OD) read at 570 nm. Cell density for each fiber on retrieval day was expressed as net growth, defined as $(OD_{\text{retrieval day}} - OD_{\text{implantation day}})/OD_{\text{implantation day,}}$ i.e. the percent change in cell density in the fibers during the trial.

The animals were observed regarding behavior and weight gain throughout the experiment. After 6 days of drug administration, 200 μl blood samples were obtained through the orbital plexus after anaesthetization with isofluran just before euthanasion by cervical dislocation. The blood samples were analyzed for hematological parameters using Micros 60 (ABX Diagnostics, Montpellier, France). Animals were caged four in each cage and fed a commercial diet (Lactamin AB, Sweden), water was given ad libitum. The study was approved by the Animal Ethics Committee in Uppsala (no. C243/6).

2.13. Statistical analysis

The IC_{50} values for FLT3 enzyme as well as for MV4-11 and 3T3L1 as measured by the flow cytometry analysis were determined using the equation $(A + ((B - A)/(1 + ((C/x)\text{^}D))))$ where A equals min, B equals max, C equals IC_{50}, D equals Hill slope and x equals compound concentration [35]. ICfix50 equals the concentration of compound resulting in 50% inhibition, in contrast to the IC_{50} value described by the equation, corresponding to the inflection point of the dose–response curve.

For the FMCA, the cytotoxic IC_{50} was determined from log concentration–effect curves in Graph Pad Prism (GraphPad software Inc., CA, USA) using non-linear regression analysis.

For comparison between groups in the in vivo hollow fiber studies, Student's t-test was used, $p < 0.05$ was considered significant.

3. Results

3.1. AKN-032 is inhibitory to the FLT3 enzyme

AKN-032 was originally identified as a hit in one of three different high throughput kinase inhibition screens of approxi-

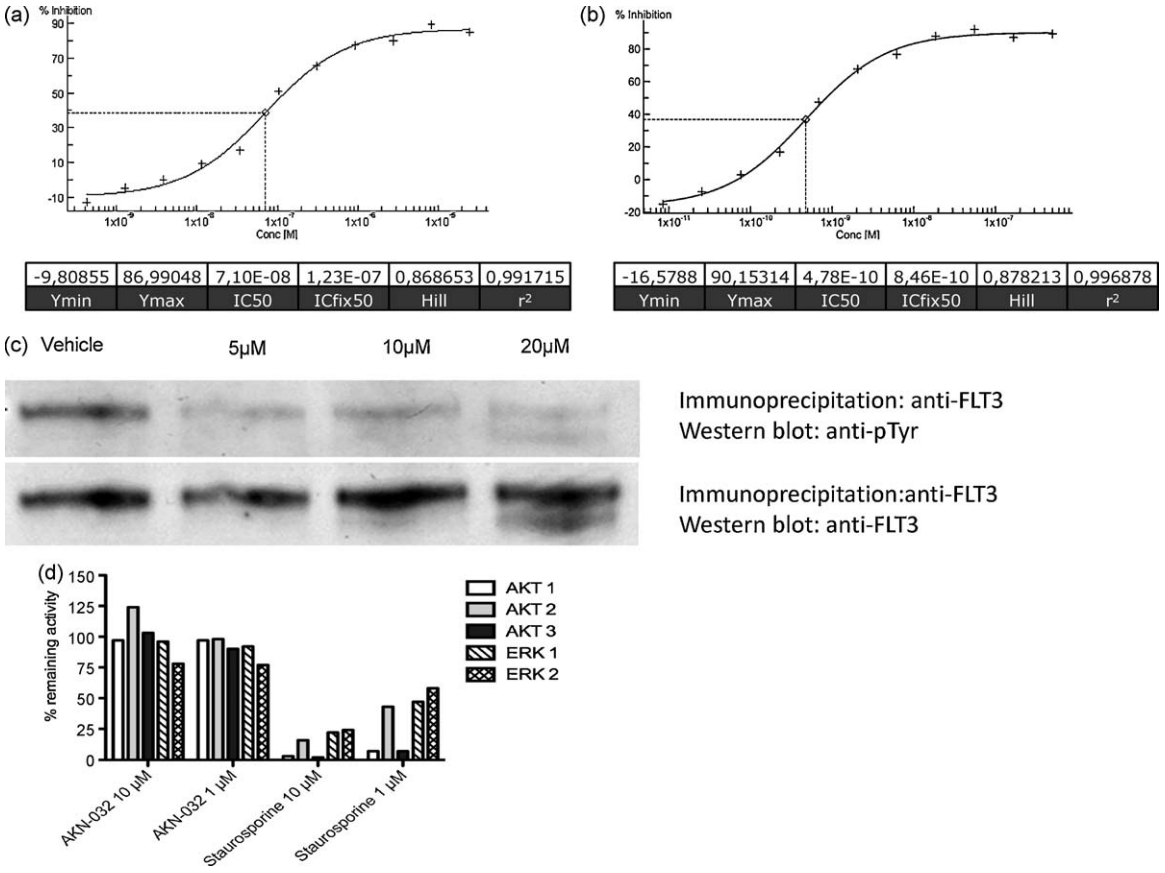


Fig. 2. AKN-032 exposure causes inhibition of FLT3 enzyme. Inhibitory activity on FLT3 enzyme of AKN-032 (a), and sunitinib (b), outlined as dose–response curves from a typical experiment in the FLT3 enzyme inhibition assay. Western blot demonstrate a reduction of FLT3 autophosphorylation in MV4-11 cells (c), after exposure to three different concentrations of AKN-032. Inhibitory activity (% remaining activity) of AKN-032 and positive control staurosporine on AKT1, 2, 3 and ERK1 and 2 at 10 and 1 μM in the radiometric protein kinase assay (d).

mately 100,000 compounds from the Biovitrum compound library, performed at Biovitrum AB. The three screens identified chemical scaffolds with FLT3-inhibitiory potential and approximately 150 compounds were selected for further analysis and verification of the FLT3-inhibitory activity. A subset of these hits, including AKN-032, was further characterized in a panel screen of 31 kinases at 10 μM. Inhibitory activity for AKN-032 was most pronounced against FLT3 kinase (data not shown). Screening data from other kinases tested (ex. PDGF-R, CK1delta, TRK A/B) indicated IC_{50} values low μM range (data not shown).

The 150 compounds identified as potentially inhibitory to FLT3 were tested in a dose–response enzyme inhibition assay. The IC_{50} value of AKN-032 on the FLT3 enzyme was determined to be 70 nM (Fig. 2a). Sunitinib was used as a positive control, typically showing an IC_{50} value of 0.5 nM (Fig. 2b), which is in accordance with data reported in the literature [36]. In addition, Western blot analysis showed that AKN-032 reduces autophosphorylation of the FLT3-receptor in MV4-11 cells (Fig. 2c).

To evaluate the effect of AKN-032 on some of the targets downstream to FLT3; the compound was also evaluated in AKT and ERK protein kinase assay. No direct inhibition of AKT1, 2, 3 or ERK1, 2 was seen at the test concentrations 1 and 10 μM (Fig. 2d).

3.2. AKN-032 is highly cytotoxic to the AML cell line MV4-11 but not to normal fibroblast 3T3-L1 cells in vitro

All compounds with an IC_{50} of 300 nM and lower in the FLT3 enzyme inhibition assay were tested in a Guava cell-kill assay against the FLT3 mutated AML cell line MV4-11. To find compounds with a preferred cell-kill profile, agents displaying activity in the MV4-11 assay were studied in counter-screen using rapidly growing normal fibroblast 3T3-L1 cells. Sunitinib and AB200434, a compound with a IC_{50} of 0.5 nM on the FLT3 enzyme [17], were used as reference compounds. Sunitinib showed cytotoxic effect on both MV4-11 and 3T3-L1, whereas AB200434 and in particular AKN-032, displayed a more MV4-11-specific activity (Table 2). Compounds highly effective against MV4-11 but lacking in toxicity against the fibroblasts were taken forward in the screening program and tested against a broader panel of tumor cell lines and primary AML samples.

3.3. AKN-032 shows selective cytotoxic activity against AML cell lines MV4-11 and Kasumi-1

Of the 15 cell lines tested in the FMCA, MV4-11 was by far the most sensitive to AKN-032 with an IC_{50} value of 0.4 μM (Fig. 3a), followed by the AML cell line Kasumi-1 (IC_{50} = 2.3 μM). The

Table 2
Inhibitory activity of AKN-032, sunitinib and AB200434 in MV4-11 and 3T3-L1 normal fibroblast cells evaluated by flow cytometry, indicated by IC_{50} values (±(SD), *n* = number of experiment repeats, N.A. = not applicable).

Compound	Cell line	IC_{50} (nM)	SD (nM)	*N*
AKN-032	MV4-11	307	103	38
	3T3-L1	>5000	NA	11
Sunitinib	MV4-11	1.5	1.2	13
	3T3-L1	2.2	0.7	6
AB240034	MV4-11	1	1.4	14
	3T3-L1	44	23.6	7

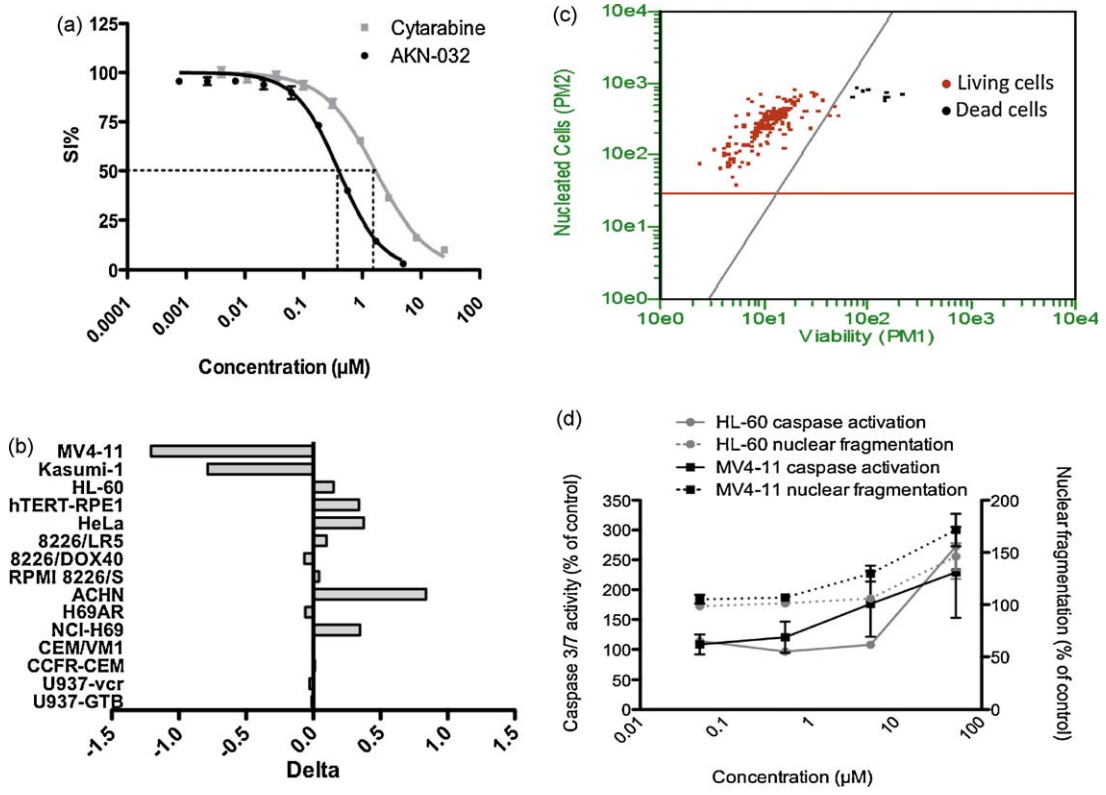


Fig. 3. AKN-032 is cytotoxic to AML cell line MV4-11. In vitro cytotoxic activity of AKN-032 and standard cytotoxic agent cytarabine measured by the FMCA in AML cell line MV4-11 ($n = 4$) outlined as dose–response curves (mean ± SEM) (a), and FMCA screening of cytotoxic activity of AKN-032 in the cell line panel displayed as delta $\log_{10}IC_{50}$ graph (b). The IC_{50} value for each individual cell line (for details – see Section 2) is converted to $\log_{10}IC_{50}$ and the mean $\log_{10}IC_{50}$ value of all cell lines is calculated. Delta is defined as the divergence of the $\log_{10}IC_{50}$ value of one cell line from the panel mean $\log_{10}IC_{50}$ value. A delta graph is constructed in which cell lines more sensitive than the average present to the left and vice versa. Scatterplot showing the difference in size and staining pattern of Guava Viability reagent from flow cytometry of MV4-11 cells after exposure to 0.6 μM of AKN-032 (c), demonstrating the presence of both living (red) and dead (black) cells. Results from caspase activation assay (d) showing dose-dependent activation of caspase activity (left axis) and nuclear fragmentation (right axis) (mean ± SEM, % of vehicle control).

corresponding IC_{50} value for the conventional AML drug cytarabine was 1.7 μM in MV4-11 (Fig. 3a). In HL-60, full cytotoxic response with no surviving cells was achieved, but at considerably higher concentrations than for the other two AML cell lines. In the other cell lines tested, including parental cell lines and drug-resistant sublines, AKN-032 displayed cytotoxic activity only at high concentrations (Fig. 3b). No difference between the parental cell lines and their respective drug-resistant sublines was observed.

Potential off-target effects were studied in a broad selectivity safety assessment panel consisting of 71 different assays for receptors, ion channels and transporters, in which AKN-032 revealed no activity (data not shown).

3.4. AKN-032 induces apoptosis in MV4-11 and HL-60 cells

The flow cytometry analysis showed the presence of dead cells even after exposure to low concentrations of AKN-032 in MV4-11 cells (Fig. 3c). To further explore the mechanism of cell death induced by AKN-032, cells from the AML cell lines MV4-11 and HL-60 were incubated for 24 h with different concentrations of the compound and then analyzed in a high content screening assay for measurement of apoptosis. Incubation with AKN-032 caused activation of caspase-3/7 accompanied by fragmentation of the nuclei (Fig. 3d), indicating that the caspase 3-pathway is active in the AKN-032-induced apoptosis in AML cell lines.

3.5. AKN-032 is cytotoxic to primary AML cells in vitro

Three of the initial 150 compounds, displaying the preferred cell-kill profile described above, were tested in the FMCA against primary AML samples. Cells from 11 AML patients; 6 mutated and 5 wild-type with respect to FLT3 (Table 1) were used, MV4-11 cells were used as positive control and sunitinib and AB200434 as reference compounds. All compounds tested were active in MV4-11, sunitinib and AB200434 being the most potent ones, but not all agents showed effect in patient cells. Primary AML cells were in general more resistant to all FLT3-inhibitors tested than MV4-11 and the IC_{50} in the cell line did not predict efficacy in patient cells. Notably, the discrepancy between the two cell systems varied depending on the compound.

Out of the compounds tested, AKN-032 was selected for further evaluation due to high cytotoxic activity in the primary tumor cells. No difference between FLT3 wild-type and mutated cases (meanIC_{50} values of 8.5 and 7.2 μM, respectively) was observed. The dose–response curve was right-shifted by 1 log unit for the primary tumor samples as compared to MV4-11 (Fig. 4a). The incongruity between cell systems for some of the other compounds, here exemplified by sunitinib, was much more pronounced in the order of 3 log units (Fig. 4b). In addition, for some compounds, here exemplified by AB200434, efficacy in MV4-11 cells did not translate to efficacy in primary AML cells (Fig. 4c). This was also the case for the two other FLT3-inhibitors from the Biovitrum compound library, displaying IC_{50}:s of 52 nM and 62 nM on the MV4-11 cells but lacking efficacy in the primary AML samples (data not shown).

3.6. AKN-032 inhibits growth of MV4-11 cells and primary AML cells in vivo

To study the pharmacokinetic and cytotoxic properties of AKN-032 in vivo, C57 black mice were given 15 mg/kg of AKN-032

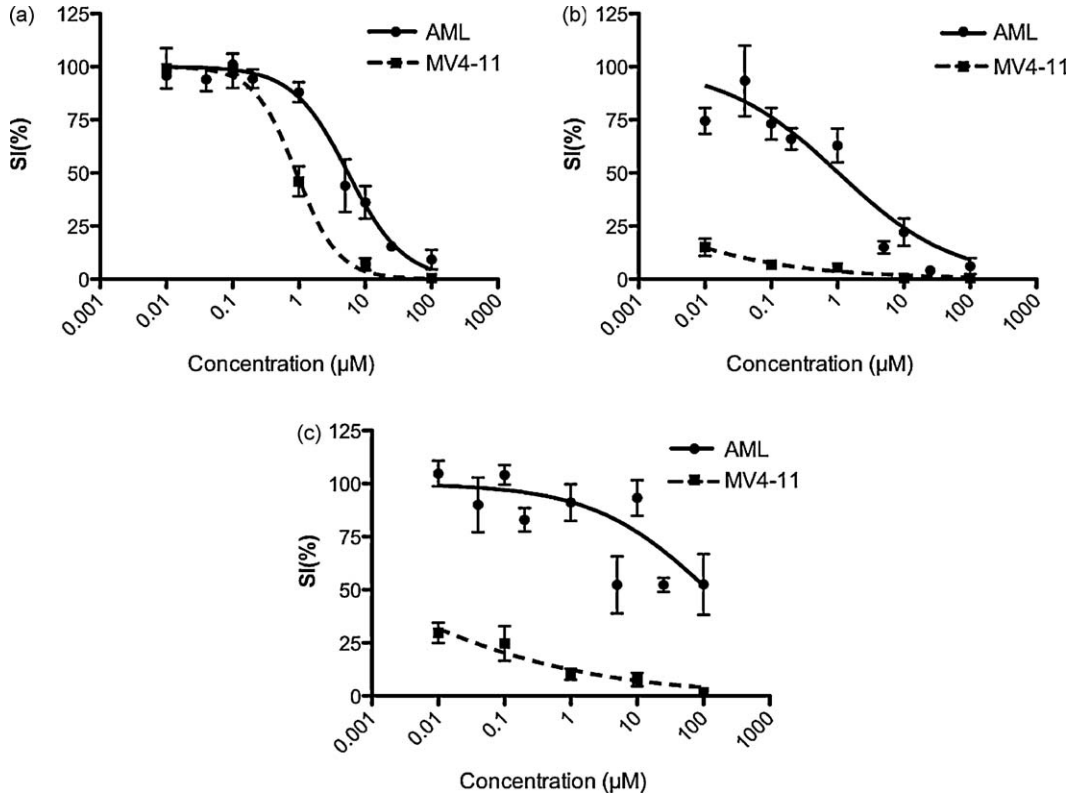


Fig. 4. AKN-032 is cytotoxic to primary AML cells in vitro. In vitro cytotoxic activity after 72 h exposure to AKN-032 (a), sunitinib (b) and AB200434 (c) in primary patient cells from AML (n = 11, mean ± SEM), measured by the FMCA, results presented as survival index (SI, %) defined as fluorescence in test wells in per cent of control cultures (cells in culture medium), blank values subtracted.

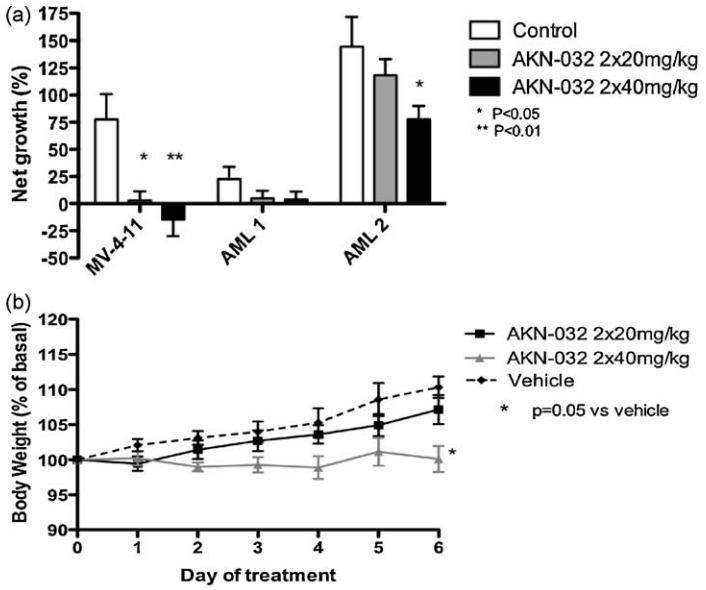
subcutaneously. The AUC was 13 μM h, maximum plasma concentration (C_{max}), was 18 μM (0.25 h after dose) and $t^1/_2$ was estimated to 0.44 h. In the hollow fiber study in NMRI mice, 6–8 fibers were available for evaluation of effect in each dosing regimen. The low dose (20 mg/kg) significantly inhibited net growth in MV4-11. At the higher dose (40 mg/kg), not only an inhibition of net growth, but a clear reduction of the leukemic mass was observed for the MV4-11 cell line (Fig. 5a). In the first AML sample, the total cell growth was very modest and no significant inhibition was observed. In the second AML sample, net growth was significantly inhibited in response to the higher dose of AKN-032 (Fig. 5a). The mice in the higher dose group of AKN-032 showed decreased food intake and reduced weight gain (p = 0.05 vs. vehicle), whereas no difference in weight gain was observed in the low dose group (Fig. 5b). As shown by blood counts analyzed on the day of fiber retrieval, no major hematological toxicity was observed in the animals (Fig. 5c). No organ effects were seen upon macroscopical inspection post-mortem.

4. Discussion

As a RTK only expressed in the hematopoetic system, with mutations and overexpression linked to disease, in particular AML, FLT3 is a valid target for drug discovery [37]. Therefore, several attempts have been made to find FLT3-inhibitors of use in the clinical setting. Focus has been on optimizing potent enzyme inhibitors with good pharmacological properties. However, clinical effects have so far been limited. As for very potent but multitarget kinase inhibitors like sunitinib or lestaurtinib, the dose required to achieve cytotoxic response through inhibition of FLT3 autophosphorylation has in some cases been associated with toxicity, limiting the clinical dose [14]. Very selective compounds, on the other hand, may not be effective in the general AML patient, but might be useful in targeting a subset of AML cells, where both proliferation and viability is dependant on high FLT3 activity [37].

In this study, we have identified a new compound, AKN-032, by using a screening funnel combining target inhibition with sequential cellular screens and aimed at identifying compounds with a preferred cell-kill profile from a subset of kinase inhibitors. In this process, the cut off level, both on enzyme inhibition and efficacy in the MV4-11 cell assay was set relatively high. About 150 compounds identified as hits in other kinase screens were selected for testing in a FLT3 enzyme inhibition assay, compounds with an IC_{50} below 300 nM in this assay were then selected for further characterization. Inhibition of MV4-11 proliferation was chosen as the first cellular assay. This cell line has been used extensively when characterizing FLT3 enzyme inhibitors both in cell-kill assays and in xenografts [25,38,39]. Compounds with an IC_{50} below 400 nM in the MV4-11 cell line were then tested in another cellular assay, using the rapidly proliferating 3T3-L1 fibroblast cell line as a counter-screen. Only compounds showing better efficacy on MV4-11 than on 3T3-L1 cells were brought to the next level of screening. The aim of this counter-screening step was to exclude compounds with a multitarget kinase inhibitory profile. Several very potent compounds, here exemplified by the reference compound AB200434, but also less potent compounds like AKN-032 displayed the preferred cell-kill profile. These compounds were then taken to the final level of cellular screening.

The aim of this project was to find compounds with selective efficacy towards AML cells with and without FLT3-mutation. Consequently, AKN-032 and two other FLT3-inhibitors from the Biovitrum compound library, as well as the two reference kinase inhibitors sunitinib and AB200434, were tested for dose–response on 11 primary patient cells: 6 harboring mutations and 5 wild-type with regards to FLT3 by using the FMCA. MV4-11 was again used as a reference cell line.



(c)

Treatment	WBC 10^9/L	RBC 10^{12}/L	HGB g/L	HCT L/L	PLT 10^9/L
Vehicle	10.8 (0.9)	9.0 (0.3)	150 (3)	0.48 (0.0)	814 (58)
AKN-032 2x20 mg/kg	11.0 (1.0)	8.9 (0.1)	154 (2)	0.48 (0.0)	753 (43)
AKN-032 2x40 mg/kg	8.2 (0.4)**	9.3 (0.3)	155 (3)	0.50 (0.0)	770 (44)

** P<0.01 vs vehicle

Fig. 5. AKN-032 inhibits growth of MV4-11 cells and primary AML cells in vivo. In vivo activity of low and higher doses of AKN-032 against AML cells (cell line MV4-11 and primary AML cells from two individual patients) in the mouse hollow fiber assay (n = 6–8) (a). Results are presented as net growth, defined as the percent change in cell density in the fibers during the 6 days of in vivo experiment (mean + SEM). Body weight development of the animals during the 6 days of the mouse hollow fiber assay (n = 6–8, mean ± SEM) (b). Hematological profile in the animals after the hollow fiber study (c). Blood samples were analyzed on the day of collection (day 6) for the following parameters: white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrite (HCT) and platelet count (PLT). Results presented as mean ± SEM.

Sunitinib showed efficacy on all tumor samples tested, the IC_{50} being three log units higher on patient cells than on the MV4-11 cell line. Surprisingly, the very potent and selective reference compound AB200434 did not show efficacy in the primary patient cells. This was also the case for the two other FLT3-inhibitors from the Biovitrum compound library with IC_{50}s of 52 and 62 nM, respectively on the MV4-11 cells, but lacking efficacy in primary patient cells. Unexpectedly, we found AKN-032, a FLT3-inhibitor with an IC_{50} of 70 nM in the FLT3 enzyme inhibition assay, inhibiting autophosphorylation of FLT3 in MV4-11 cells and with a relatively high IC_{50} in the MV4-11 flow cytometry cell proliferation assay, to be more potent on the primary patient cells than for example AB200434. Thus AKN-032 fulfilled the criteria set out in our screening funnel, despite not being the most potent compound.

In a cell line panel representing several different hematological and non-hematological malignancies, the newly synthesized tyrosine kinase inhibitor AKN-032 showed significant cytotoxic activity in the AML cell lines MV4-11 and Kasumi-1 as measured by the FMCA. In the third AML cell line tested HL-60, a cell line reported to be FLT3 negative [25], a full cytotoxic response was achieved, but at noticeably higher concentrations.

As expected, IC_{50} for AKN-032 in the FMCA cell-kill assay was higher than in our FLT3 kinase assay. This may be due to pharmacokinetic factors related to the cellular models used, including uptake and efflux of the compound (data not shown). Still, the dose–response curves from the MV4-11 experiments followed a sigmoid pattern with an IC_{50} of 0.4 μM, a value lower than the results observed for the AML standard drug cytarabine in the same experiment. Exploration of death characteristics show that the AKN-032-induced apoptosis in AML cell lines MV4-11 and HL-60 at least in part has its origin in the activation of caspase-3/7.

MV4-11 is an excellent model system for FLT3 mutated AML [40], but as a cell line, it is likely to have acquired additional genetic changes in becoming immortalized and adapted for continual growth [41]. Although being essential for investigation on mechanism of action of cytotoxic drugs and valuable tools in drug discovery, cell lines may have limitations when it comes to predicting clinical outcome [42,43]. Primary patient tumor cells, on the other hand, provide a model well suited for preclinical prediction of tumor-specific activity and direction of phase II trials to suitable patients [44]. This prompted us to include efficacy on primary patient cells in our screening funnel, including both FLT3 mutated and wild-type AML samples. Our results confirmed that AKN-032 has significant in vitro activity in AML. The more prominent effect (i.e. lower IC_{50}) in the cell line assay reflects a high proliferation rate making the cells more sensitive to cytotoxic drugs in general. The effect of AKN-032 in rapidly growing cell lines like MV4-11 is caused by a combination of absent proliferation and cell death, as shown by the Guava ViaCount assay as well as the caspase activation assay. In contrast to cell lines, most primary AML tumor cells are low proliferating in vitro and the efficacy of AKN-032 in these cells may to a greater extent be caused by cell death.

In our primary tumor material, we observed no difference in sensitivity to AKN-032 between FLT3 mutated and wild-type AML cases. There are findings suggesting a role of the wild-type FLT3-receptor in AML [9], others proposing that a variance in quantitative expression of wild-type FLT3, regardless of mutation status, might explain different response to AKN-032 as well as to other putative FLT3-inhibitors [45]. Results from the AKT and ERK protein kinase assay show no direct inhibition of these kinases. This along with the cytotoxic effect of AKN-032 in the reported FLT3 negative AML cell line HL-60 might suggest additional mechanisms of cytotoxic activity of the compound. Studies

investigating a possible correlation between levels of wild-type FLT3 expression and the response to AKN-032 as well as other mechanisms of action in AML are underway.

The hollow fiber assay allows studies of the effect of different substances in vitro as well as in vivo. Using the hollow fiber method in animal models makes it possible to study pharmacokinetics, tumor effects and hematological toxicity of anticancer drugs in the same animal [34]. Thus, we were able to demonstrate an antitumoral effect in this relatively resistant model, suggesting a good distribution of AKN-032 to subcutaneous tissue in mice. Importantly, no significant toxic effects were observed, however the animals in the higher dose group showed a reduction in weight gain. We speculate that this may be related to nausea with decreased food intake as a result.

As for pharmacokinetics, subcutaneous injections of AKN-032 in C57 black mice resulted in a short time to peak concentration and relatively short $t^1/_2$. Assuming linear pharmacokinetics and no strain differences, one can predict that twice daily injections in NMRI mice in the hollow fiber model result in a similar exposure to the compound to that observed in C57 black mice.

In conclusion, AKN-032 is a new tyrosine kinase inhibitor with significant in vitro cytotoxic activity in several different AML cell types, seemingly irrespective of FLT3-mutation status. Results from the hollow fiber mouse assay demonstrate in vivo activity and suggest a favorable toxicity profile of the drug. Future preclinical studies will focus on pharmacokinetic properties and toxicity, as well as further clarifying the mechanisms of action of AKN-032 in AML.

Conflict of interest

The following authors were employees of Biovitrum AB when these experiments were performed or when this manuscript was written and revised: C. Ekholm, K. Fhölenhag, A. Jenmalm Jensen, A. Löthgren, M. Scobie, V. Parrow. V. Parrow is now primary affiliated to and part owner of Akinion Pharmaceuticals AB. M. Scobie is primary affiliated to Orexo AB and A. Åleskog works as medical advisor for Merck Sharp and Dome AB. C. Ekholm is a minor stockholder of Biovitrum AB.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.08.002.

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