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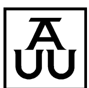
Studies of New Signal Transduction Modulators in Acute Myeloid Leukemia

ANNA ERIKSSON



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Abstract

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Acute myeloid leukemia (AML) is a life-threatening malignant disorder with dismal prognosis. AML is characterized by frequent genetic changes involving tyrosine kinases, normally acting as important mediators in many basic cellular processes. Due to the overexpression and frequent mutations of the FMS-like receptor tyrosine kinase 3 (FLT3) in AML, this tyrosine kinase receptor has become one of the most sought after targets in AML drug development.

In this thesis, we have used a combination of high-throughput screens, direct target interaction assays and sequential cellular screens, including primary patient samples, as an approach to discover new targeted therapies. Gefitinib, a previously known inhibitor of epidermal growth factor receptor and the two novel tyrosine kinase inhibitors AKN-032 and AKN-028, have been identified as compounds with cytotoxic activity in AML.

AKN-028 is a potent inhibitor of FLT3 with an IC_{50} value of 6 nM in an enzyme assay, but also displaying in vitro activity in a variety of primary AML samples, irrespective of FLT3 mutation status or quantitative FLT3 expression. AKN-028 shows a sequence dependent in vitro synergy when combined with standard cytotoxic agents cytarabine or daunorubicin, with better efficacy when cells are exposed to standard chemotherapy simultaneously or for 24 hours prior to adding AKN-028. Antagonism is observed when cells are pre-treated with AKN-028, possibly explained by the cell cycle arrest induced by the compound. In vivo cytotoxic activity and good oral bioavailability have made AKN-028 a candidate drug for clinical studies and the compound is presently investigated in an international two-part multicenter phase I/II study.

Results from microarray studies performed to further elucidate the mechanism of action of AKN-028, revealed significantly altered gene expression induced by AKN-028 in both AML cell lines and in primary AML cells, with an enrichment of the Myc pathway among the downregulated genes.

Furthermore, tyrosine kinase activity profiling shows a dose-dependent kinase inhibition by AKN-028 in all AML samples tested. Interestingly, cells with a high overall kinase activity were more sensitive to AKN-028. Provided conformation in a larger set of samples, kinase activity profiling may give useful information in individualizing treatment of patients with AML.

Keywords: Acute myeloid leukemia, Targeted therapies, Drug development, Tyrosine kinase inhibition, AKN-032, AKN-028

Anna Eriksson, Uppsala University, Department of Medical Sciences, Cancer Pharmacology and Computational Medicine, Akademiska sjukhuset, SE-751 85 Uppsala, Sweden.

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”Cancer therapy is like beating a
dog with a stick to get
rid of his fleas”

- Anna Deavere Smith, *Let me down easy*

List of Papers

This thesis is based on the following Papers, which are referred to in the text by their Roman numerals.

I Lindhagen E, **Eriksson A**, Wickström M, Danielsson K, Grundmark B, Henriksson R, Nygren P, Åleskog A, Larsson R, Höglund M. Significant cytotoxic activity in vitro of the EGFR tyrosine kinase inhibitor gefitinib in acute myeloblastic leukaemia. *European Journal of Haematology.* 2008 Nov;81(5):344-53.

II **Eriksson A,** Höglund M, Lindhagen E, Åleskog A, Hassan SB, Ekholm C, Fhölenhag K, Jenmalm Jensen A, Löthgren A, Scobie M, Larsson R, Parrow V. Identification of AKN-032, a novel 2-aminopyrazine tyrosine kinase inhibitor, with significant preclinical activity in acute myeloid leukemia. *Biochemical Pharmacology.* 2010 Nov 15; 80(10):1507-16

III **Eriksson A**, Hermanson M, Wickström M, Lindhagen E, Ekholm C, Jenmalm Jensen A, Löthgren A, Lehmann F, Larsson R, Parrow V, Höglund M. The novel tyrosine kinase inhibitor AKN-028 has significant antileukemic activity in cell lines and primary cultures of acute myeloid leukemia. *Blood Cancer Journal.* 2012 Published online Aug 3

IV **Eriksson A,** Kalushkova A, Jarvius M, Hilhorst R, Rickardson L, Göransson Kultima H, de Wijn R, Fryknäs M, Öberg F, Larsson R, Parrow V, Höglund M. AKN-028 induces cell cycle arrest, downregulation of Myc-associated genes and a dose dependent reduction of kinase activity in acute myeloid leukemia. *Manuscript*

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Abbreviations

ALL	Acute lymphocytic leukemia
Allo-SCT	Allogeneic stem cell transplantation
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
ATRA	All-trans retinoic acid
CLK1	CDC-like kinase 1
CR	Complete remission
DNMT3	DNA (cytosine-5) methyltransferase 3
EGFR	Epithelial growth factor receptor
FLT3	FMS-related tyrosine kinase 3
FLT3-ITD	Internal tandem duplication of FLT3
FLT3-TKD	Tyrosine kinase domain mutation of FLT3
FLT3-wt	Wild type FLT3
FMCA	Fluorometric microculture cytotoxicity assay
GSEA	Gene set enrichment analysis
IC_{50}	Half maximal inhibitory concentration
IDH 1/2	Isocitrate dehydrogenase
JAK	Janus Kinase
LSC	Leukemic stem cell
MDR	Multidrug resistance
NF-κB	Nuclear factor kappa B
NPM1	Nucleophosmin 1
OS	Overall survival
PARP	Poly ADP-ribosylation polymerases
PDGFR	Platelet derived growth factor receptor
PI3K	Phospoinositide 3-kinase
mTOR	Mammalian target of rapamycin
MAPK	Mitogen-activated protein kinase
NCI	National Cancer Institute
RPS6K	Ribosomal protein S6 kinase
SI	Survival index (%)
STAT	Signal transducer and activator of transcription factor
TET2	Tet methylcytosine deoxygenase 2
TKI	Tyrosine kinase inhibitor
UPN	Unique patient number
WHO	World Health Organization

Introduction

Cancer biology

Cancer describes a large and heterogeneous group of diseases characterized by the uncontrolled multiplication and spread of abnormal forms of the body's own cells. Carcinogenesis is a multistep process where normal cells acquire accumulated mutations over time. Hallmarks of cancer include uncontrolled proliferation and unlimited replicative potential as well as evasion of apoptosis. Furthermore, cancer cells loose the spatial respect of the surrounding tissues that healthy cells maintain to enable normal continuous growth, thereby making cancers invasive and prone to metastasize[1,2]. In addition, epigenetic modulation of gene expression, i.e. modifications occurring without alterations in the DNA sequence itself, is another key characteristic of cancer[3].

Alterations in oncogenes promoting malignant development, or mutations inactivating tumor suppressor genes that are supposed to protect from tumor formation, can both cause cancer[4]. The vast majority of cancer mutations damage somatic cells, and only alterations in the DNA of germ cells will pass on to the next generation, making most cancers a genetic disease on a cellular level[1,5].

Cell cycle control

The irreversible, ordered sets of events that characterize the cell cycle are not only critical for regulation of cell division and growth in general, but also for cell division after DNA damage[6,7]. Most cells in adults are not in the process of cell division, but rest in a quiescent state (G_0) outside the division machinery. Mitogens or growth factors can release the cells from G_0, thereby introducing them into the first gap phase (G_1) during which the cell prepare for DNA replication. However, before this passage, cells must pass through the G_1 restriction point, but once they do so, cells are irreversibly committed to progress through the cell cycle. G_1 is followed by the synthetic phase (S), in turn succeeded by the second gap phase (G_2) and subsequent mitosis (M), the latter two with their own checkpoints aiming to maintain the integrity of the genome. After the completed cell division, the two new daughter cells have the option of either become inactive in G_0, or to re-enter the cell cycle

through G_1[1,8,9]. Deregulation of this intricate system and mutations in its regulators are common features in human malignancies[9,10].

Cell death

Apoptosis is a highly regulated process of programmed cell death with an important role in both normal development and in eliminating damaged DNA. Since the introduction of the concept of programmed cell death in the 1960s, apoptosis has been considered the "clean and tidy" way of cell death, whereas necrosis has been regarded to represent a messy, unorganized mechanism leading to spillage of intracellular components and inflammation[1,11-13]. However, recent evidence somewhat shifts this belief by revealing that necrosis can also occur in a regulated fashion[14,15].

According to accepted models, two non-exclusive pathways can be used to induce apoptosis, both using specific proteases; caspases, as key-players in the signaling cascade. The intrinsic apoptotic pathway (mitochondria-initiated pathway) responds to a wide range of stimuli inside the cell, leading to the activation of the caspase 9/3 cascade as well as several caspase–independent cell death effectors. The extrinsic pathway, on the other hand, is mainly activated by ligand binding to a transmembrane cell death receptor, leading to a conformational change in the receptor and subsequent intracellular signaling through initiator caspase 8 and the executioner caspases 3, 6 and 7[1,12,15].

Evasion of apoptosis is one of the key features of neoplasms and normal cells can become malignant as a consequence of mutations in the apoptotic machinery. One of the important goals in the constantly evolving apoptosis research field has been the development of new and better cancer treatments[16].

The leukemic stem cell

Understanding of the normal hierarchical path for an undifferentiated stem cell to a committed progenitor cell, subsequently giving rise to a fully differentiated cell, is a prerequisite to gain insight to cancer development. Because of their constitutional capacity for self-renewal, allowing accumulation of genetic changes, the early stem cells have often been favored candidates as the target of malignant transformation[5].

The first evidence for the concept of cancer stem cells was reported in patients with acute myeloid leukemia (AML) and results from xenogenic transplant models have shown that a very small number of leukemic blast cells can engraft and restore a leukemic clone, fueling the hypothesis of leukemic stem cells (LSC)[17,18]. Classification of cells can be made by detection of cell surface markers and the LSC, like its normal stem cell counterpart, is usually found in the $CD34^+/CD38^-$ compartment, although LSC have been

found in a $CD34^-$ subset in patients with nucleophosmin 1 (NPM1)-mutated AML[19,20]. The original hypothesis set forth that the LSC had its origin in a primitive stem cell compartment undergoing malignant transformation through a series of mutations [17]. Alternate theories suggest that LSC could also arise from more committed progenitors due to changes in gene expression, leading to enhanced self-renewal capacity[21,22].

Like normal stem cells, LSC divide slowly and are often quiescent, a fact possibly underlying the ineffectiveness of standard chemotherapy, which exercise much of its action in cells actively proliferating in the cell cycle[23]. Recent gene expression analysis of AML subpopulations with LSC activity, revealed a LSC-associated signature highly predictive for poor clinical outcome, a finding possibly useful in defining patients with increased risk of relapse[18,24].

Leukemia

Annually, around 50 000 new cases of cancer are detected in Sweden. Although prognosis differs considerably between the around 200 different subtypes, tumor disease account for nearly 25% of all deaths in the country, making it the second most common cause of death. Around 1% of Swedish cancer cases consist of leukemia, and hematological malignancies, including lymphomas, are the third most common cancer related cause of death in the country[25].

Following several case descriptions, the term leukemia, derived from Greek and literally meaning “white blood”, was taken to use by Rudolf Virchow in the 19th century. The term describes a group of different malignant disorders originating in the hematopoietic system and affecting the white blood cells. Leukemias are divided into myeloid or lymphoid depending on the origin of the cell type primarily affected, and these groups, in turn, are subdivided into chronic and acute leukemias depending on the stage of maturity of the malignant cells[26,27].

Acute myeloid leukemia - AML

Acute myeloid leukemia (AML) is an aggressive disease, or rather a diverse group of disorders, characterized by clonal expansion of early hematopoietic blast cells (see Figure 1). The abnormal accumulation of immature cells displaces the normal hematopoiesis, thereby causing bone marrow failure[28,29]. Approximately 330 Swedish adults are diagnosed with AML annually. The disease can present in all ages but mainly in the elderly, with a median age of 72 years and a peak incidence at 80-84 years[30]. Intense chemotherapy will induce a complete remission (CR, defined as no morphologically detectable

disease and recovery of white blood cell and platelet counts[31]) in up to 80% of patients. However, despite consolidating therapy, sometimes including allogeneic stem cell transplantation (allo-SCT), a majority of patients will ultimately relapse. Furthermore, patients with high age, secondary AML or those who are unfit for intense chemotherapy, rarely obtain longstanding remissions[32]. Presently, 40-50% of patients < 60 years of age are cured, whereas elderly patients have a 5-year survival of less than 15%[30,31,33].

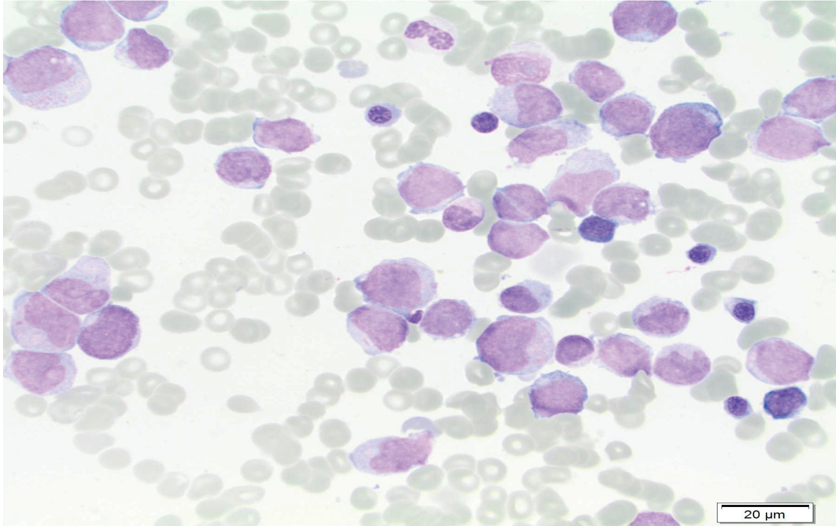

Figure 1. Acute myeloid leukemia blast cells, courtesy of Dr. Rose-Marie Amini

Classification and prognostic factors

In 1976, the French-American-British (FAB) co-operative group presented the first consentient classification of AML, based on morphology, cellularity, blast percentage and cytochemistry, dividing AML into eight subtypes (M0-M7)[34]. In 2001, the World Health Organization (WHO) introduced a new classification, including many of the FAB criteria but expanded to use all available information in the diagnosis, including morphology, genetic, immunophenotypic and biologic features as well as clinical characteristics[35]. A revised classification was presented in 2008[36,37] and is described in Table 1. For the diagnosis of AML, a bone marrow or peripheral blast count of ≥ 20% is required, with the exception of certain cases carrying special recurrent genetic aberrations[36].

Table 1. WHO classification of Acute Myeloid Leukemia[36]

Acute myeloid leukemia with recurrent genetic abnormalities
t(8;21)(q22;q22); RUNX1/RUNX1T1*
inv(16)(p13q22) or t(16;16)(p13;q22); CBFB/MYH11*
t(15;17)(q22;q21); PML/RARA*
t(9;11)(p21;q23); MLLT3/MLL
t(6;9)(p22;q34); DEK/NUP214
inv(3)(q21q26) or t(3;3)(q21;q26); RPN1/EVI1
t(1;22)(p13;q13); RBM15/MKL1
Provisional entity: AML with mutated NPMI
Provisional entity: AML with mutated CEBPA
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified
Acute myeloid leukemia with minimal differentiation
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute myelomonocytic leukemia
Acute monoblastic and monocytic leukemia
Acute erytroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferation related to Down's syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemia of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemia with t(9;22)(q34;q11); BCR/ABL1
Mixed phenotype acute leukemia with t(v;11)(v;q23); MLL-rearranged
Mixed phenotype acute leukemia, B/myeloid
Mixed phenotype acute leukemia, T/myeloid
Provisional entity: Natural killer (NK)-cell lymphoblastic leukemia/lymphoma

* The diagnosis of AML can be established without regard to blast cell count

Survival is closely linked to the risk of relapse and the goal of treatment is therefore to achieve and sustain a CR. Prognostic factors can be subdivided into patient-related, leukemia-related and therapy-related factors[31,38].

Patient-related risk factors

Increasing age is a well-known risk factor, both on its own but also caused by the fact that elderly AML patients more often display chemotherapy resistance or high-risk cytogenetics. Furthermore, aged patients are more likely to have poor performance status or significant comorbidities[39,40].

Leukemia-related risk factors

The cytogenetic properties of the leukemia remains the cornerstone in predicting prognosis in AML and the karyotype provides a framework for current risk stratification[41,42]. Patients are generally categorized into three risk groups based on cytogenetics; favorable, intermediate and adverse (presented in Table 2)[31,42]. Patients in the favorable group have a CR rate above 90% with an overall survival (OS) of 55-85%, whereas patients in the adverse risk group have a CR rate of only 60% and a gloomy OS of about 10-20% or in elderly patients as low as 5%[29,33,43,44]. These two prognostic groups are relatively easy to adapt to a risk stratification plan trying to decide whether patients are candidates for an allo-SCT in first remission or not. Remaining is the largest and extremely heterogeneous intermediate group containing patients with normal or nonconclusive karyotypes.

In recent years, a wide range of molecular prognostic factors have been identified in AML and the mutation status of the CCAAT/enhancer binding protein α gene (CEBPA), NPM1 gene and the FMS-related tyrosine kinase 3 gene (FLT3) are currently used for treatment stratification[45]. The field of molecular genetics is rapidly expanding and new technology constantly reveals novel genetic aberrations, possibly allowing classification of AML into an increasing number of subtypes[46-48]. Currently, more than 90% of AML patients can be categorized based on either cytogenetic or molecular genetic features[42]. Aside from the previously described subgroups, several other leukemia-related risk factors can occur, sometimes in covariance, thereby making them more difficult to evaluate as independent variables. Secondary and therapy-related AML have been reported to be associated with reduced CR rate as well as impaired OS[49,50]. Furthermore, hyperleukocytosis is a risk factor for early complications and death in remission induction[51].

Table 2. Genetic aberrations correlated to AML prognosis[31]

Prognostic group	Genetic aberration
Favorable	t(8;21)(q22;q22); RUNX1-RUNX1T
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	Mutated NPM1 without FLT3-ITD (normal karyotype)
	Mutated CEBPA (normal karyotype)
Intermediate-I	Mutated NPM1 and FLT3-ITD (normal karyotype)
	Wild-type NPM1 and FLT3-ITD (normal karyotype)
	Wild-type NPM1 without FLT3-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); MLLT3-MLL
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
	t(6;9)(p23;q34); DEK-NUP214
	t(v;11)(v;q23); MLL rearranged
	-5 or del(5q); -7; abnl(17p); complex karyotype

Therapy-related risk factors

Response to initial treatment and achievement of CR are strong independent prognostic factors for survival. Patients with >15 % blast counts after the first treatment or those who need more than two courses of chemotherapy to obtain CR have impaired long-term survival[52-54]. Remaining minimal residual disease (≥ 0.1%) detectable by flow cytometry has also been linked to higher relapse rate and shorter survival[55-57].

Clinical signs and symptoms

Patients often present with a history of repeated infections, profound anemia, pallor, dyspnea and lethargy. Thrombocytopenia gives rise to easy bruising and increased frequency of bleedings and neutropenia causes susceptibility to infections. Leukemic infiltration of tissues such as liver, spleen or skin represent more unusual manifestations of AML[29]. Contrary to acute lymphocytic leukemia (ALL), involvement of the central nervous system at time of diagnosis is rare[58].

AML treatment

Despite great effort to stratify patients, AML first line treatment is still very uniform, with the exception of acute promyelocytic leukemia (APL). The standard treatment is based on a combination of cytotoxic agents, traditionally anthracyclines such as daunorubicin or idarubicin and the pyrimidine analogue cytarabine, the latter also used as a consolidating agent[31,59]. In treatment of relapse, various combinations of standard chemotherapeutics have been used (Table 3). Allo-SCT is the most efficient way to eradicate persisting leukemic cells and thereby prevent relapse. The strength of this method lies in the so-called "graft-versus-leukemia" effect, an immunological response from donor T-cells eliminating possible remaining leukemic cells. Unfortunately, the benefit of an allo-SCT is limited by the fact that the reactive T-cells can also attack the normal tissues of the recipient in a so-called "graft-versus-host" reaction[33,60].

Table 3. Standard chemotherapeutic agents used in AML treatment[2,61]

Chemotherapeutic Agent	Drug Class	Mechanism of action
Daunorubicin	Anthracycline, Cytotoxic antibiotic	Inhibitor of DNA synthesis by intercalation between base pairs. Topoisomerase II inhibition. Single and double strand breaks.
Idarubicin	Anthracycline, Cytotoxic antibiotic	Inhibitor of DNA synthesis by intercalation between base pairs. Topoisomerase II inhibition. Single and double strand breaks.
Mitoxantrone	Topoisomerase II inhibitor	DNA binding causes strand break. RNA reaction. Topoisomerase II inhibition.
Cytarabine	Antimetabolite, Pyrimidine analogue	Enters target cell and undergoes phosphorylation reaction as the physiological nucleoside. Incorporated in both RNA and DNA but main cytotoxic action through inhibition of DNA polymerase.
Amsacrine	DNA-synthesis inhibitor	Not entirely clear, inhibits DNA synthesis, not RNA
Etoposide	Topoisomerase II inhibitor	DNA damage, inhibition of mitochondrial function and nucleoside transport. Topoisomerase II inhibition.
Fludarabine	Antimetabolite, Purine analogue	Metabolized to triphosphate. Inhibition of DNA synthesis similar to cytarabine
Azacitidine*	Hypomethylating agent	Inhibition of DNA, RNA and protein synthesis

* MDS-AML with 20-30% blast count

Signal transduction in AML

Genetic changes involving tyrosine kinases, important regulators in basic cellular processes such as proliferation, survival and differentiation, are frequent in leukemia. Deregulation of signaling pathways, including those involving tyrosine kinase signaling, have been connected to leukemogenesis and progression of leukemic disease, thereby making them attractive targets in antileukemic drug discovery[62,63]. In 2002, the hypothesis of AML being a multistep process where at least two classes of mutations have to pool resources to initiate the disease, was launched[64]. This model of leukemogenesis suggest a collaboration between a class I mutation that gives the cells a proliferation advantage through activation of signal transduction pathways, and a class II mutation impairing cell differentiation by affecting transcription factors. Subsequently, unclassified mutations including those related to epigenetic regulation (e.g. DNA (cytosine-5) methyltransferase 3 A (DNMT3A)) have also been linked to leukemic development[64,65].

The receptor tyrosine kinase FLT3 is predominantly expressed on normal early multipotent hematopoietic cells and plays an important role in the regulation of proliferation, differentiation and apoptosis of hematological progenitor cells[66]. In AML, FLT3 is commonly expressed at high levels on the leukemic blasts. FLT3 signaling affects downstream targets in several pathways such as the phospoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), RAS/mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription factor 5 (STAT5)[64,67,68]. During the last decade, FLT3 has been one of the prime targets in directed drug discovery in AML[68,69].

Activating mutations of FLT3 are among the most common genetic alterations in AML, affecting approximately 30% of patients. Two major groups of activating FLT3 mutations have been described in AML patients: internal tandem duplications in the juxtamembrane domain (FLT3-ITD), leading to constitutive kinase activation[70,71] and tyrosine kinase domain point mutations (FLT3-TKD)[72]. The vast majority of mutations are FLT3-ITD, which has been associated with increased relapse rate and reduced overall survival. However, the prognostic significance of FLT3-TKD mutations is still not fully elucidated[70,72,73].

Along with FLT3, other signal transducers have emerged as possible targets for drug development. Thus, aberrantly expressed KIT, RAS, platelet derived growth factor receptor (PDGFR) and Janus kinases (JAK) have been reported to play a role in leukemogenesis[74-76]. KIT mutations are detected in approximately 20-30 % of AML patients with t(8;21) or inv(16) and are associated with impaired prognosis. Patients harboring a KIT mutation together with either t(8;21) or inv(16) have a cumulated 5 year relapse incidence of 70 and 80% respectively, compared to 30-35% relapse rate for patients with the same cytogenetic abnormality but lacking KIT alterations[28,77].

The RAS signaling pathway is another important regulator in the proliferation and survival of hematopoietic progenitors. Oncogenic RAS mutations cause constitutive activation in up to 25 % of AML patients[75,78]. Apart from direct mutations, the RAS pathway can also be affected by upstream tyrosine kinase receptors such as FLT3 and KIT, contributing to its activation[79].

Drug discovery and development

Drug discovery and development of new anticancer drugs is a complex process, largely depending on established tumor cell lines and animal in vivo tumor models. Unfortunately, the correlation between activity in these model systems and actual clinical activity in patients is not perfect, and clinical phase I–II studies are therefore often performed as a kind of arbitrary screening in patients, normally at a late stage disease[80-82].

Anticancer drug development dates back to the 1940s with the discovery and launch of nitrogen mustards and antifolate drugs. Since the 1950s, the National Cancer Institute (NCI) in the United States has hosted a systematic drug screening program and for the first thirty years, most screenings were carried out in vivo using murine P388 or L1210 leukemia cell lines. The 1980s brought about a new disease-oriented screening, utilizing a panel of 60 cell lines covering a wide range of tumor types. The rapidly progressing understanding of the signaling networks regulating cellular activities such as proliferation and survival has identified new potential drug targets such as growth factors, signaling molecules, cell cycle proteins and effectors of apoptosis and angiogenesis. This explosion of basic cancer knowledge has in large shifted drug discovery towards targeted therapies[83,84].

Development of targeted therapy

Classical anticancer agents mainly work by targeting DNA and cell replication. This relatively blunt strategy is afflicted with poor biochemical selectivity, severe side effects and the development of drug resistance. The growing understanding of the mechanisms and pathways engaged in cell signaling during the start of the twenty-first century has created a rationale for a more target based drug discovery approach[4,83]. The list of success stories include inhibition of the tyrosine kinase Bcr/Abl by imatinib mesylate (Gleevec®) in chronic myeloid leukemia[85] and the effect of the monoclonal antibody trastuzumab (Herceptin®) in breast cancer overexpressing the human epidermal growth factor receptor 2 (HER2)[86]. Furthermore, many other targeted compounds such as monoclonal CD20 antibody rituximab (Mabthera®) in lymphomas, epithelial growth factor receptor (EGFR)-inhibitors erlotinib (Tarceva) and gefitinib (Iressa) in lung cancer and the proteasome targeting

drug bortezomib (Velcade®) in myeloma, have served as proof of concept for this strategy[4,87].

Targeted therapy has also proven its place in AML. APL is a specific subtype of AML classified by the balanced translocation t(15;17), causing the subsequent expression of fusion protein PML/RARα that serves as an aberrant transcription factor interfering with myeloid differentiation. APL has a specific morphology and is clinically characterized by disseminated intravascular coagulopathy and high incidence of early fatal hemorrhages[88]. The introduction of all-trans retinoic acid (ATRA), directed against the aberrant protein causing the differentiation block, has revolutionized the treatment for APL patients. ATRA induces terminal differentiation and when combined with conventional chemotherapy, a complete remission rate of 90-95 %, thereby shifting the prognosis from highly fatal to strikingly curable[89,90].

Signal transduction modulation – general aspects

Tyrosine kinases have emerged as some of the most interesting drug targets in AML and other hematological malignancies. A wide variety of methods to establish kinase profiles for new compounds are presently in use. As reviewed by Uitdehaag et al., profiling studies can be divided into three basic categories: 1) Dose-response binding experiments giving K_d values (indicating strength of binding) for each target 2) Dose-response experiments determining the half maximal inhibitory concentration (IC_{50}) for each target kinase and 3) Measurement of a single inhibitor concentration, revealing %-inhibition effect or %-remaining activity[91]. A common technique is to start with screening at a fixed concentration to narrow down the possible targets, followed by IC_{50} determination for the most interesting hits. To date, 15 small-molecule kinase inhibitors have been approved, all but one for treatment of various forms of cancer, ranging from highly selective to multi-target inhibitors[92].

The question of what constitutes a good kinase inhibition profile is not finally settled as both selective and multi-kinase inhibitors have their pros and cons. Thus, very few compounds are strictly selective for a single target and most agents display a degree of promiscuity by their very nature. Selective drugs offer the advantage of limited off-target related toxicity but are on the same time associated with the risk of resistance as the whole efficacy relies on one target. Multi-kinase inhibitors represent the other side of the spectrum with the chance of higher efficacy at the expense of safety[92-94]. The latest trend in this research field is found somewhere in between, searching for drugs acting in a "selectively nonselective" manner; safe but with the potential to benefit many patients[92,93].

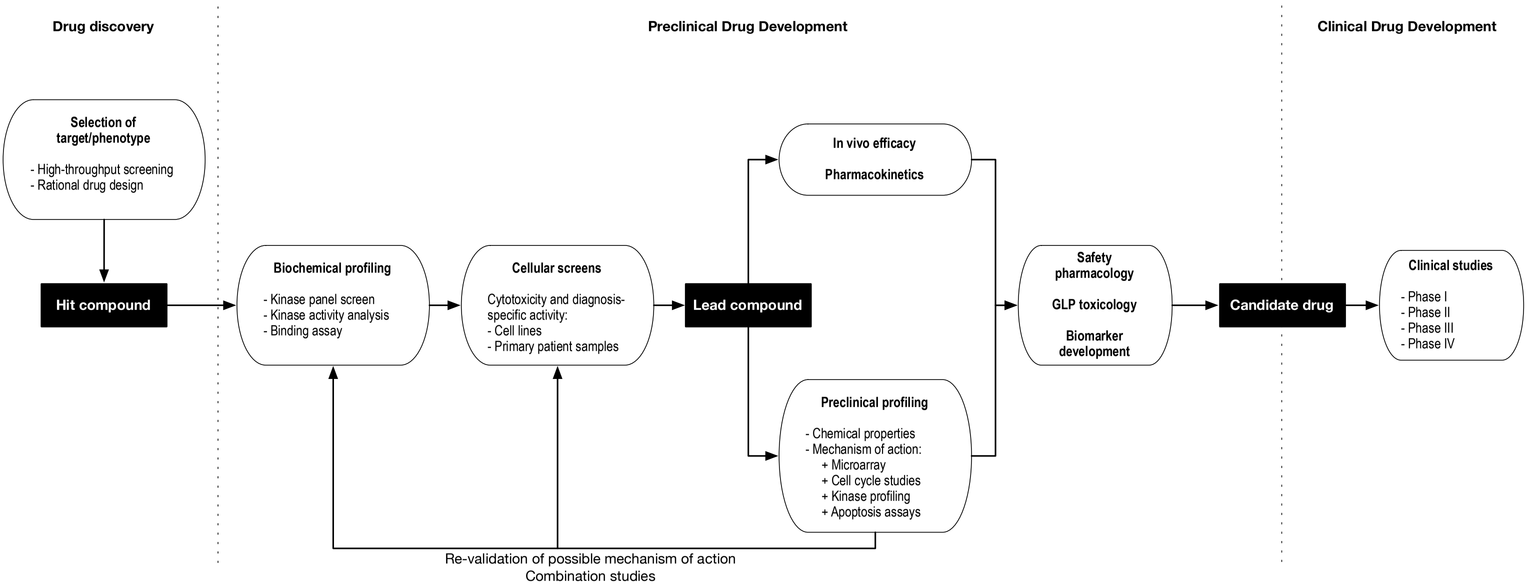

Figure 2. Development plan for targeted anticancer drug development, exemplified by kinase inhibitors.

Practical approach to targeted drug discovery

The development plan for a new targeted anticancer agent includes multiple steps, starting with the identification of a target or phenotype. The next step involves identification of a lead compound, commonly through either high-throughput screens of thousands of compounds or rational drug design. The screening hits are taken forward to biochemical profiling and target validation, followed by sequential cellular screens utilizing tumor cell lines and sometimes primary tumor samples, for evaluation of cytotoxic and diagnose-specific activity. Lead compounds are further evaluated for in vitro efficacy and the compound emerging as the candidate drug is taken forward through additional in vivo testing including pharmacokinetics, formulation studies and assessment of toxicity. In parallel, the candidate drug is also profiled with regards to chemical properties and mechanism of action[4,80,95,96]. An example of a development plan used in the drug discovery of kinase inhibitors is presented in Figure 2.

Cell lines and primary tumor samples

Tumor cell lines represent a monoclonal population of immortalized cells adapted to continual growth in culture without undergoing senescence. Cell lines constitute an essential corner stone in drug discovery with their practically unlimited supply of homogenous cell material to be used for evaluation of for instance cytotoxic activity or modeling of drug resistance[87,97]. Although crucial for many aspects of anticancer research, cell lines are likely to have acquired additional genetic changes in becoming immortalized and have limitations when it comes to prediction of clinical outcome[81,98]. Primary tumor samples present an alternative in vitro model system for compound screening and is well adapted for preclinical prediction of tumor specific activity and direction of early clinical trials to suitable patients[99].

Animal models

Animal models are used in the drug development process to confirm in vitro observations made in cell lines or primary tumor samples in the presence of a host response. Furthermore, animals are used in the evaluation of pharmacokinetic properties and toxicology. The most extensively used efficacy models for hematological malignancies are human xenografts in immune-deficient mice and genetically engineered models.

Tumor xenografts are generated by transplantation of tumor cells into immune-deficient mice, unable to reject the malignant cells. Xenograft tumors can be ectopic (subcutaneously established) or orthotopic (transplantable into their original tissue)[4,80,100,101].

Transgenic and gene targeted animals are generated by addition of an exogenous DNA vector construct into the genome. Although expensive and time consuming, transgenic models allow the tumors to develop in a setting

within a host with an intact immune system and more accurately mimic the natural malignant development[4,80,102]. The hollow fiber method, developed by the NCI to bridge between in vitro based assays and xenograft models, utilizes semipermeable fibers filled with human tumor cells (described in detail in the Material and Methods section)[103].

Despite their usefulness in many aspects of anticancer drug development, animal models also have their limitations. Due to both ethical and economic reasons, these models are not well suited for high-throughput screenings. In addition, it is very difficult to create an adequate model system recapturing the heterogeneity and great genetic diversity of most tumors. Furthermore, due to more rapid metabolism, higher tolerance for side effects and divergence in protein binding, animals do not perfectly mirror humans with regards to pharmacokinetic properties[83,87].

Novel therapies in AML

Although targeted therapy is in focus in modern antileukemic drug discovery, there is still progress in the field of more conventional chemotherapeutic agents. Clofarabine (Evoltra®) is a second generation nucleoside analogue. Clofarabine was rationally designed to overcome the limitations of its predecessors fludarabine and cladribine, but also to incorporate the best qualities of these compounds. For example, clofarabine is halogenated at the 2-position of adenine, making the compound resistant to intracellular enzymatic degradation. Furthermore, increased stability to acidic environment is another of the improved properties of clofarabine. Clofarabine is approved for third line treatment in ALL but has shown efficacy in AML as well[104,105]. Elacytarabine is a lipophilic 5'elaidic acid ester of cytarabine representing another novel nucleoside analogue that recently showed effect in a phase II study in advance stage AML[106].

Signal transduction modulation

Due to the overexpression and relatively frequent mutations of FLT3 in AML, this tyrosine kinase receptor has been one of the most sought after targets in AML drug development. FLT3 belongs to the same subfamily of receptor tyrosine kinases as PDGFR, KIT and vascular endothelial growth factor receptor (VEGFR). These receptors share some structural homology, which might explain why FLT3 inhibitors often have activity against the rest of the members of the subfamily as well[67,107,108].

A number of tyrosine kinase inhibitors (TKIs) have shown promising activity against FLT3 both in vitro and in vivo. Members of the first generation of FLT3 inhibitors, including the staurosporine analogues lestaurtinib (CEP-701) and midostaurin (PKC-412), were multi-targeted compounds. However, although effective in inhibiting FLT3 autophosphorylation, preventing blast proliferation and promote apoptosis, several of these agents are limited by

suboptimal pharmacokinetics and insufficient specificity[109-111]. Thus, in Phase I-II trials, the single agent activity of lestaurtinib and midostaurin was limited and of short duration. Theoretically more promising, both these compounds have also been tested in combination with standard chemotherapy[112]. However, the combined treatment with lestaurtinib and chemotherapy failed to show a survival advantage with the addition of lestaurtinib but a higher incidence of adverse events[113]. Results from the phase Ib study combining midostaurin with standard chemotherapy have recently been published, but final results from the ongoing prospective randomized double-blinded phase III study are still awaited[114]. Sorafenib is another kinase inhibitor with effect on FLT3, which in small studies has shown both single agent activity and complete remissions when combined with chemotherapy in FLT3-mutated AML[115].

Of the second generation FLT3-inhibitors, AC220 (quizartinib) has shown very selective FLT3-activity both in vitro and in vivo. Moreover, AC220 has a pharmacokinetic profile with very long plasma half-life and have shown promising results in early clinical trials including some complete responses in a heavily pre-treated patient group[109]. Follow-up studies with AC220 in combination with chemotherapy are in progress[115 110,116].

Other tyrosine kinases, such as KIT and PDGFRB, have been suggested as possible targets for AML drug development, but results from early clinical trials are still inconclusive. Considering that AML is a highly heterogeneous disease, it is likely that patients will have a better chance to benefit from a more synergistic approach combining new kinase inhibitors with other compounds, either conventional chemotherapeutic agents or newer targeted drugs[62,117].

Epigenetic modulation

Epigenetics describe changes in gene expression through other mechanisms than changes in the actual DNA sequence. In recent years, there has been a rapid progress in the understanding of epigenetic modification in the initiation and progression of cancer. The findings of global hypomethylation of DNA, hypermethylation of tumor suppressor genes and DNA methylated and inactivated micro-RNA in human cancers, have made epigenetics a prime target in anticancer drug development[3,118,119].

Recently, several mutations in genes linked to epigenetic regulation have been identified in AML, such as the Tet methylcytosine deoxygenase 2 (TET2) gene, which encodes for a protein serving as an enzyme in the process of DNA demethylation. TET2 mutations, which have been linked to impaired enzymatic function, are detected in 10-20% of AML cases and although the prognostic value is still unclear, an adverse impact on leukemia-free survival has been suggested[42,120,121]. In normal karyotype AML, mutations in the genes encoding isocitrate dehydrogenase (IDH1 and 2) have been reported at a relatively high frequency. Interestingly, patients with

IDH1/2 and TET2 mutations show a similar epigenetic signature and global DNA hypermethylation. DNMT3A, normally serving as a methyltransferase that generates DNA methylation, is mutated in 14-18% of AML cases and at an even higher rate in normal karyotype AML. The role of DNMT3A in leukemogenesis is still not fully elucidated, but mutations of this methyltransferase have been associated with impaired prognosis in some studies[42,120,121]. The fact that DNA methylation and histone modification, unlike mutations, are reversible processes has made epigenetic regulators attractive drug targets. Consequently, the DNMT-inhibitors azacitidine (Vidaza®) and 5-aza-2'-deoxycytidine (Dacogen®), as well as the histone deacetylase inhibitor vorinostat (Zolinza®) have been developed for treatment of myelodysplastic syndrome and cutaneous T-cell lymphomas respectively. In AML, both azacitidine and 5-aza-2'-deoxycytidine have proven useful, although their precise role in AML treatment is still not clear[3,121].

Immune modulation

Gemtuzumab ozogamicin (GO; Mylotarg) is an anti-CD33 monoclonal antibody conjugated to the toxin calicheamicin. Since CD33 is frequently expressed on leukemic blasts, the toxicity of GO mainly affects these tumor cells[122]. GO gained FDA-approval but was later withdrawn from the market when a study combining GO with standard chemotherapy failed to improve overall survival and was associated with a significantly higher risk of induction mortality[123]. Later studies combining GO with conventional chemotherapy have shown survival benefits in certain subgroups of AML patients, primarily those with favorable cytogenetics, thereby possibly warranting a comeback for the compound[122,124].

Interleukin 2 (IL-2) is a T-cell derived cytokine suggested as immunotherapy in AML since it induces both T-cell and natural killer (NK) cell response to tumor cell targets[125]. Histamine dihydrochloride (Ceplene®) has been shown to potentiate the IL-2 effect in vitro. Post-consolidating therapy with the combination has been related to significantly improve leukemia-free survival, especially in FAB-subgroups M4 and M5[126].

Modulation of apoptosis and drug resistance

Evasion of apoptosis is one of the hallmarks of cancer. In addition, this process has known connections to drug resistance. Bcl-2 is an apoptosis inhibitor protein that when overexpressed makes tumor cells resistant to induction of apoptosis. As high levels of bcl-2 in AML have been reported as a potential mechanism for drug resistance as well as linked to poor prognosis, bcl-2 has been pursued as a potential drug target using agents such as antisense oligonucleotides and small molecular inhibitors[127-129]. Another strategy to induce apoptosis is exemplified by the novel compound APR-246, which restores the function of p53. Recently, APR-246 has been tested in a clinical trial in patients with hematological malignancies or prostate cancer[130].

Poly ADP-ribosylation polymerases (PARPs) are important players in the post-translational modifications of nuclear proteins after single or double-stranded DNA damage. Upregulation of PARP activity helps malignant cells evade apoptosis induced by DNA damaging therapy. The potential of PARP inhibition to sensitize cells to chemotherapy has made it an interesting target in AML[129].

Expression of plasma membrane glycoprotein (Pgp) is closely linked to multidrug resistance (MDR). More commonly expressed in elderly patients, as well as in cases with relapsed or chemorefractory AML, high expression of Pgp has been linked to inferior prognosis. Although very appealing as a treatment strategy, results from hitherto tested MDR-modulators have not been very encouraging[63,127,129].

Modulation of the ubiquitin-proteasome pathway

The function of the ubiquitin-proteasome pathway is to dispose intracellular proteins, including important mediators of the cell cycle and apoptosis, such as cytokines, caspases, nuclear factor kappa B (NF-κB) and bcl-2. Since malignant cells are highly proliferative, they are exceedingly dependent on this system to degrade the large amount of proteins produced[131]. Thus, the reversible proteasome inhibitor bortezomib, established for treatment of multiple myeloma, is currently being investigated in AML. Thalidomide and its derivate lenalidomide, both drugs probably acting through multiple mechanisms including inhibition of NF-κB, are being tested in various hematological malignancies including AML[63,132].

Inhibition of the molecular chaperone heat shock protein 90 (Hsp90) is a recently introduced targeted therapy strategy with actions that can be placed under several of the subheadings in this chapter. Hsp90 is expressed at high levels in AML, especially in FLT3-ITD AML, probably due to the fact that misfolded proteins generated by mutations to a higher degree require chaperoning. Inhibition of Hsp90 leads to FLT3 polyubiquitination and proteasomal degradation[66,133,134].

Aims

The overall aim of this thesis was preclinical evaluations of new signal transduction modulators in acute myeloid leukemia. The specific aims of the studies were the following:

- To evaluate the compounds gefitinib, AKN-032 and AKN-028 regarding in vitro cytotoxic activity in hematological malignancies as well as in normal hematological cells, including mechanisms of the cytotoxic response/cell death.
- To investigate potentially synergistic drug interactions of gefitinib and AKN-028, respectively, when combined with conventional antileukemic agents.
- To investigate the kinase inhibiting activity of AKN-032 and AKN-028.
- To investigate whether the cytotoxic response to AKN-032 and AKN-028 differs between FLT3-wild type and FLT3-mutated leukemic cells.
- To study if there is a correlation between the in vitro effect of AKN-028 and the level of quantitative FLT3 expression in primary AML samples.
- To further explore the mechanism of action of AKN-028 through investigation of cell cycle effects, tyrosine kinase profiling as well as changes in gene expression in primary AML cells and tumor cell lines after exposure to AKN-028.
- To study AKN-032 and AKN-028 in mice regarding cytotoxic response and toxicity profile.

Material and Methods

This section is a summary of experimental methods used in this thesis, for further details see the respective Papers I-IV.

Primary patient samples and cell culture

Throughout this thesis, tumor cell lines and primary tumor samples obtained from bone marrow, peripheral blood, routine surgery or diagnostic biopsy of adult patients with different malignancies, as well as normal peripheral blood mononuclear cells (PBMC), were used for evaluation of cytotoxicity and mechanistic studies. Leukemic cells and PBMC were isolated by density gradient centrifugation, whereas cells from solid tumor samples were isolated by combined mechanical and collagenase dispersion followed by Percoll gradient centrifugation. All cells were cryopreserved in -150 °C and thawed at experimental setup. Cell viability was determined by trypan-blue exclusion test and the proportion of tumor cells was determined by inspection of May-Grünwald-Giemsa stained cytocentrifuge preparations[135]. The sampling was approved by the Ethics Committee of Uppsala University (No 21/93 and 2007/237).

A previously described panel of cell lines[136] was expanded to include five AML cell lines (described in detail in Papers II and III) and used for investigations of diagnose-specific activity of compounds. Throughout this thesis, the FLT3-ITD mutated AML cell line MV4-11 has been used as a model system.

Drugs

In Paper I, we focused on gefitinib (Iressa®), a low-molecular weight anilinoquinazoline compound originally described as an inhibitor of the tyrosine kinase EGFR. Through collaboration with Biovitrum AB, and later on with Akinion Pharmaceuticals, we gained access to a number of new signal transduction modulators, where the 2-aminopyrazine tyrosine kinase inhibitors AKN-032 and AKN-028 were identified as the most promising ones (Papers II, III and IV). Conventional chemotherapeutic agents were used for comparison, as well as five other tyrosine kinase inhibitors: imatinib (Paper I),

sunitinib and midostaurin (with broad kinase inhibition profiles, Papers II, III & IV) and AB200434 and AC220 (with a more FLT3-specific effect, Papers II and III).

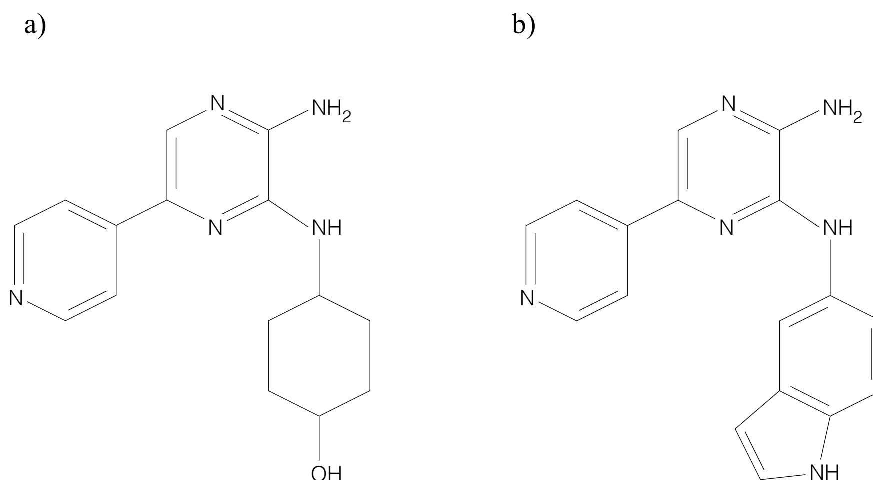

Figure 3. Chemical structures of AKN-032 (a) and AKN-028 (b).

Cytotoxicity assays

Three cytotoxicity assays were used; the fluorometric microculture cytotoxicity assay (FMCA), the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide-assay (MTT) and flow cytometry.

The Fluorometric Microculture Cytotoxicity Assay

The cytotoxic activity of most compounds was evaluated by use of the fluorometric microculture cytotoxicity assay (FMCA), described in detail previously[137]. This in vitro method is based on the measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes. Microplates containing cell suspension were incubated with serially diluted compounds.

Living cell density was assessed after 72 hours by measurement of the generated fluorescence after 50 minutes of incubation with FDA. Fluorescence is proportional to the number of intact cells and results are presented as survival index (SI, %) defined as fluorescence in test wells in percent of control cultures, blank values subtracted. Quality criteria to accept assay results included fluorescence signal in control cultures >5x of mean blank values, the mean coefficient of variation in control cultures < 30% and tumor cell fraction surpassing 70% after incubation.

MTT-assay

For evaluation of effect in the hollow fiber experiments, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)-assay[138] was used. The method is based on the conversion of MTT to blue formazan crystals by living cells. Dimethyl sulfoxide (DMSO) can extract formazan and the optical density (OD) can be read at 570 nm. Cell density for each fiber on retrieval day is presented as net growth (the percent change in cell density, defined as $(OD_{retrieval\ day} - OD_{implantation\ day}) / OD_{implantation\ day}$.

Flow cytometry- viability assay

In Paper II, the effect of TKIs on viability was analyzed using flow cytometry. Serial dilutions of the TKIs made in culture medium supplemented with DMSO were transferred to cell culture plates containing MV4-11 or 3T3-L1 cells and then incubated for 72 hours.

Viability reagent (Guava ViaCount) was added to each well and number of cells and viability was determined using Guava 96-well ViaCount assay. Percent survival was calculated compared to vehicle treated cells at the end of the experiment.

Drug combination analysis

In Papers I and III, TKIs were studied in combination with standard chemotherapeutic agents. Combinations were designed with a fixed molar ratio between agents, intended to be equipotent, and both simultaneous and sequential addition of drugs were applied. Living cell density was assessed by the FMCA as described above. Possible interactions between agents in the study were analyzed as proposed by Chou and Talalay[139] by median effect analysis using the CalcuSyn software (Biosoft, Cambridge, UK).

Apoptosis assays

Two different methods were used to study the cell death characteristics of cells exposed to gefitinib, AKN-032 or AKN-028.

Image-based apoptosis assay

In Papers I and II, AML cells were seeded into 96-well plates, exposed to different concentrations of TKI and evaluated by a multiparametric single-cell assay[140]. Probes staining activated caspase-3 and nuclei were added and

caspase activity and nuclear fragmentation was analyzed in the ArrayScan® high content screening system (Cellomics Inc, Pittsburgh, PA, USA).

Live-cell imaging of apoptosis

In Paper III, the cell death characteristics of AKN-028 were studied by identification of caspase-3 by use of the live-cell imaging instrument IncyCyte (Essen BioScience Ltd, Welwyn Garden City, UK), providing a kinetic readout of apoptotic signal. A caspase inhibitor was added as a control.

Cell cycle analysis

Analysis of cell cycle distribution in Paper IV was performed as described by Vindeløv et al[141]. The method estimates cell cycle distribution from cell nuclei DNA content and based on detergent lysis of cell membranes with a subsequent staining with proprium iodide, a red fluorescent dye that binds to DNA enabling quantification of the nuclei by flow cytometry.

FLT3 mutation detection and quantitative FLT3 assay

Primary AML samples were examined regarding FLT3 mutation status; genomic DNA was analyzed by polymerase chain reaction (PCR) followed by capillary electrophoresis for fragment analysis to detect FLT3-ITD and genomic DNA was analyzed by PCR to detect FLT3-TKD[73]. In Paper III, an assay to evaluate total quantitative expression of FLT3 was developed. Total RNA was isolated from primary AML cells and reverse-transcribed into cDNA. The FLT3 mRNA transcripts were quantified by quantitative RT-PCR, and the analysis measured the overall expression of both wild type and mutant FLT3 transcripts. A standard curve for FLT3 was created using the plasmid pCDHF3 for wild type FLT3 (FLT3-wt)[142]. GUSB was used as reference gene and the amount of FLT3 transcripts was expressed as a ratio of FLT3 copy number relative to 100 GUSB copies.

Kinase activity analysis

Several techniques have been used in this thesis to establish kinase activity of different compounds. In Papers II and III, the kinase inhibitory profiles of AKN-032 and AKN-028 were evaluated at fixed concentrations over two commercially available kinase assessment panels as well as a broad selectivity safety assessment panel. Further evaluation of the most interesting targets has been made in full dose-response experiments. An enzyme inhibi-

tion assay, where the kinase domain was incubated with a fluorescent peptide substrate and the generated fluorescence was measured, was used to evaluate FLT3 kinase activity after drug exposure with AKN-032 or AKN-028. The semi-quantitative method western blot was used for evaluation of inhibition of FLT3 autophosphorylation in Papers II and III. In Paper III, the more quantitative phospho-ELISA method was used to detect inhibition of FLT3 and KIT autophosphorylation. Furthermore, a radiometric protein kinase assay was used for measurement of kinase activity of AKT1, 2, 3 and ERK1, 2.

Pamchip peptide microarrays

In Paper IV, tyrosine kinase activity profiles were determined using the PamChip® tyrosine peptide microarray system (PamGene International B.V. ‘s-Hertogenbosch, the Netherlands). This method is based on measurement of peptide phosphorylation on an array with kinase peptide substrates immobilized inside a porous membrane made of aluminum oxide[143]. Cell lysates treated with AKN-028 were run through the array by an up and down movement of the solution in the automated work station PamStation®12, maximizing the opportunity for kinases to phosphorylate the 144 peptides on the array. The functional readout is based on kinetic measurement of the phosphorylation on the array by use of fluorescently labeled antibodies. Quality control and data analysis was performed using the Bionavigator software.

Expression of EGFR – immunohistochemistry

In Paper I, samples from AML patients as well as the AML cell line MV4-11 were analyzed by immunohistochemistry with regards to expression of EGFR. Cell preparations were incubated with a primary anti-EGFR antibody for 30 minutes at room temperature, followed by incubation with a secondary antibody, enzyme conjugate and a chromogen[144].

Hollow fiber assay

The hollow fiber model was developed by the NCI as a tool for screening of anti-cancer drugs, by growing human tumor cells inside semi-permeable polyvinylidene fluoride hollow fibers and subjecting them to different compounds[103]. The method enables studies of pharmacokinetics, tumor effect and hematological toxicity in both cell lines and primary patient samples in vivo, within the same immunocompetent animal[145], a fact that in turn reduces the number of animals needed. The hollow fiber method is relatively resistant,

with a risk of underestimating drug effect due to low efficacy of drug delivery to the subcutaneously implanted fibers. The study was approved by the Animals Ethics Committee in Uppsala (No. C243/6).

Pharmacokinetics

The pharmacokinetic properties of compounds AKN-032 and AKN-028 were evaluated in mice after subcutaneous, oral and intravenous administration. Blood samples were collected at nine time points under the first 24 hours following administration and plasma concentrations of the drugs determined by protein precipitation and liquid chromatography-electrospray ionization tandem mass spectrometer. The study was approved by the Animal Ethics Committee in Northern Stockholm (No. N94/04 and N57/07).

Gene expression analysis

Changes in gene expression induced by treatment with AKN-028 were investigated in two AML cell lines and in cells from one AML patient using microarray technique (Affymetrix GeneChip® arrays). Leukemic cells were seeded into 6-well plates, grown for 24 hours and then exposed to AKN-028 or reference compound midostaurin for 6 hours. Cells were washed with phosphate buffered saline (PBS) and RNA preparation and microarray analysis were performed as previously described[146]. Subsequent analysis of gene expression data was performed as described in Paper IV. To further characterize of the mode of action of AKN-028, a gene set enrichment analysis (GSEA) was performed using the generated microarray results. GSEA utilizes predefined sets of genes, connected by for instance common biological features, pathways or proximity in the genome. The predefined gene sets are run though a ranked gene-list to reveal possible enrichment at the extremes. This run through the rank list will calculate an enrichment score, mirroring the maximum deviation from 0 (which would represent a randomly distributed gene set). Since the enrichment score will depend on the size of the gene set, a normalization to account for this is made[147].

Statistics

The half maximal inhibitory concentration (IC_{50}) was defined as the concentration giving a SI-value of 50%. The relative IC_{50} corresponds to a response midway between the estimates of the lower (min) and the higher (max) plateau of a dose-response curve[148]. With control values defining min and max, we searched for compounds able to achieve full efficacy (i.e. 0% cell surviv-

al), and therefore calculated IC_{50} values only for curves with stable upper and lower plateaus ranging approximately between 0 and 100%. The IC_{50} was determined from log concentration–effect curves in Graph Pad Prism (GraphPad software Inc., CA, USA) using non-linear regression analysis or determined using the equation (A+((B-A)/(1+((C/x)^D)))) where A equals min, B equals max, C equals IC_{50}, D equals Hill slope and x equals compound concentration.

Comparison of activity between two different groups was made with Student's t-test or Mann-Whitney U-test and correlations were investigated by Spearman's rank order correlation. A p-value of <0.05 was considered significant.

Results and Discussion

In vitro off target activity of EGFR tyrosine kinase inhibitor gefitinib in AML (Paper I)

Significant in vitro cytotoxic activity of gefitinib in AML

Gefitinib (Iressa®) is a low-molecular weight anilinoquinazoline compound originally described as an inhibitor of the tyrosine kinase EGFR, and the compound is currently in use for treatment of non-small cell lung cancer. Hematological malignancies do not express EGFR, but several publications suggest that gefitinib may act also by non-EGFR dependent mechanisms. Moreover, there are reports on gefitinib promoting differentiation and decreased viability of EGFR-negative AML blasts[149].

In Paper I, we investigated the activity of gefitinib in different tumor cells, in particular in AML. The cytotoxic activity, alone and in combination with standard cytotoxic agents, was evaluated in tumor cells from 117 patients representing both hematological and non-hematological malignancies. Gefitinib was found to have significant cytotoxic activity in AML, and when combined with conventional chemotherapy additive or synergistic effects were observed. Gefitinib induced apoptosis in all four primary AML samples and in the AML cell line MV4-11 when tested in a multiparametric high content screening assay, even though AML cells did not express EGFR (as determined by immunohistochemistry). Our conclusion is that gefitinib exercise significant cytotoxic activity in AML by inducing apoptosis through non-EGFR dependent pathways. The actual mechanism of action remains to be elucidated, although a recent study have implied a role for Src-family kinases and the tyrosine kinase Btk as direct targets for gefitinib, possibly mediating the cytotoxic effect[150]. A phase II study investigating the role of gefitinib in patients with relapsed or refractory AML has been performed (Clinicaltrials.gov NCT00130702) but results from this study are still awaited.

Identification of the novel tyrosine kinase inhibitor AKN-032 with significant preclinical activity in acute myeloid leukemia (Paper II)

Introduction of a new screening funnel

The aim of Paper II was to use a new screening funnel combining targeted based approach with cellular screens in trying to identify compounds with efficacy in AML cells without targeting healthy cells. More than 100 000 compounds of the Biovitrum's Compound library were subjected to three high-throughput screens directed against serine and threonine kinases. Approximately 150 compounds with FLT3 inhibitory potential were evaluated at a fixed concentration (10 µM) over a panel of 31 kinases, followed by assessment in a dose-response FLT3 enzyme inhibition assay to establish the IC_{50} values. Reduction of autophosphorylation of the FLT3-receptor was analyzed by Western blot. Only compounds with an IC_{50} value <300 nM in the FLT3 enzyme assay were propelled forward to a cellular counter-screen, trying to identify agents with high activity in leukemic cell line MV4-11 but not in normal fibroblast 3T3-L1 cells. Only compounds with the preferred cell kill profile were investigated for diagnose-specific activity in a panel of 15 cell lines. In parallel, compounds were tested for potential off target effects in a panel of 71 receptors, ion channels and transporters. If displaying AML-specific activity without hits in the off target panel, compounds were taken to the final step of the screening funnel and investigated for effect in primary AML samples, both FLT3 wild type and mutated cases. The set up and selection criteria of the screening funnel is summarized in Figure 4.

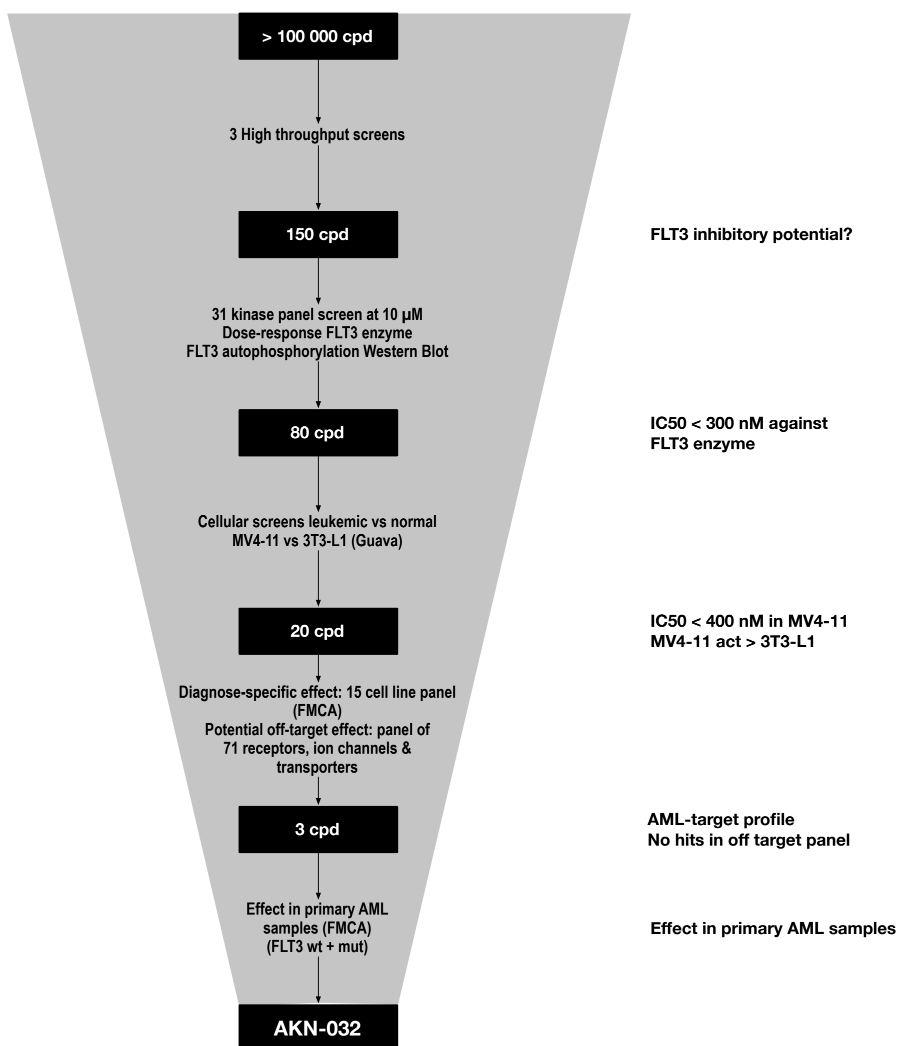

Figure 4. Screening funnel used to investigate >100 000 compounds (cpd).

Antileukemic activity of AKN-032 in AML

AKN-032 fulfilled all the criteria set out in the screening funnel. The compound inhibits FLT3 enzyme activity with an IC_{50} value of 70 nM, reduces autophosphorylation of FLT3 receptor in the AML cell line MV4-11 and is cytotoxic to MV4-11 (IC_{50}: 307 nM) but not to normal fibroblast cells (IC_{50}: >5000 nM). Furthermore, when AKN-032 was tested against a cell line panel and against primary tumor samples from AML patients, the compound showed a diagnose-selective cytotoxic activity against AML cells.

Finding the optimal kinase inhibition profile is a challenging task. Whether one chooses a broad-spectrum inhibitor or a selective profile, both approaches have their drawbacks. In this study, we used two different compounds with a wide variety in kinase selectivity as drug references: the multi-targeted kinase inhibitor sunitinib[151], at the time the study was performed under investigation in AML, and the FLT3 specific inhibitor AB200434[152]. Multi-target compounds like sunitinib, lestaurtinib and midostaurin have the disadvantage of off target toxicity and the dose required to accomplish cytotoxic response through reduction of FLT3 autophosphorylation has in some cases produced dose-limiting toxicity[153,154]. Kinase selective compounds might however be too narrow to be of benefit for the general AML patient[92].

Sunitinib was effective in both AML cells and normal fibroblasts. Although highly cytotoxic against MV4-11 cells, the IC_{50} value was approximately 1000 times higher in patient cells than in the MV4-11 cell line. On the contrary, AB200434 was highly effective in MV4-11 but lacked dose-dependent response in primary AML samples. AKN-032 was not as potent as the reference compounds on FLT3 enzyme or against MV4-11 cells, but nevertheless showed a good cytotoxic response in primary AML cells.

The lack of correlation between IC_{50} values in cell lines and primary patient samples is an interesting methodological finding. Part of the right-shift in dose response curves might be explained by a difference in proliferation rate between cell lines and primary patient cells, or alternatively by a higher dependency of FLT3 in the former cell type. Which model system ultimately is the best predictor for actual clinical activity in AML patients can be debated, but it might be good to keep in mind that potent activity in one model system not necessarily translates in to the next one.

AKN-032 was further evaluated in mice with respect to pharmacokinetic properties as well as antileukemic activity. AKN-032 was cytotoxic to MV4-11 cells and primary patient cells in vivo and displayed a favorable toxicity profile, thereby strengthening the conclusion that AKN-032 is a new tyrosine kinase inhibitor with promising effect in AML.

AKN-028 – a novel tyrosine kinase inhibitor with significant activity in AML (Paper III)

Identification of AKN-028, a potent FLT3 and KIT inhibitor

In Paper III, we presented the tyrosine kinase inhibitor AKN-028, identified in a screen of approximately 400 analogues of the previously reported AKN-032 (Paper II). AKN-028 is a potent FLT3 inhibitor with an IC_{50} of 6 nM in an enzyme assay and the compound initiates a dose-dependent inhibition of FLT3 autophosphorylation. Fixed concentration screening at 1 μM in a 320

kinase panel revealed < 20% remaining activity only for FLT3, ribosomal protein S6 kinase (RPS6K) and CDC-like kinase 1 (CLK1). In the phospho-ELISA assay used, AKN-028 also inhibited KIT autophosphorylation. FLT3 and KIT belong to the same kinase subfamily and share many common features, and many FLT3 inhibitors also targets KIT[67]. The discrepancy between results from the kinase panel screen and the phospho-ELISA may result from the fact that the former only mirrors the active form of KIT, whereas the latter reflects both the active and the inactive form of the kinase. In a panel of 17 cell lines, AKN-028 showed cytotoxic activity in all five AML cell lines included and was furthermore shown to induce apoptosis in MV4-11 by activation of caspase 3.

AKN-028 is cytotoxic to primary AML cells, irrespective of FLT3 mutation status or quantitative FLT3 expression

The cytotoxic activity of AKN-028 was evaluated in primary AML samples, showing a dose-dependent response in all samples tested, irrespective of FLT3 mutation status ($p = 0.79$, Student's t-test). Dose-response curves for FLT3-wt and FLT-ITD samples obtained from the FMCA are presented in Figures 5 a and b respectively. The lack of correlation between cytotoxic activity and FLT3 mutation status raised the question whether it was in fact not the mutation itself but rather the quantitative expression of FLT3 that was the key to the response to FLT3 inhibitors. A method to measure the overall quantitative FLT3 expression was set up, but revealed no correlation between antileukemic activity and FLT3 quantitative expression or to the allelic burden (Figures 5 c+d). These findings warrant further investigations regarding the mechanism of action of AKN-028 in AML.

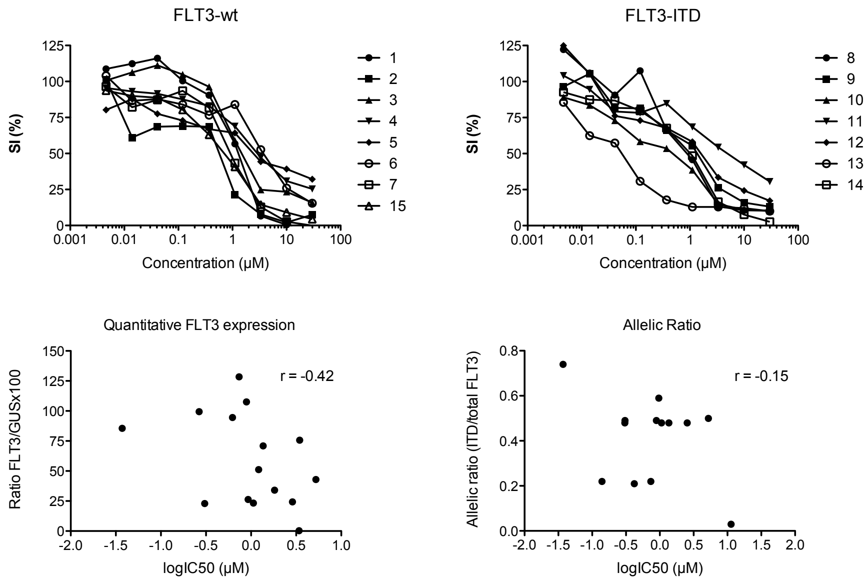

Figure 5. Cytotoxic response to AKN-028 in vitro displayed as dose-response curves in FLT3-wt (a) and FLT3-ITD (b) primary AML samples as well as logIC50 values plotted against quantitative FLT3 expression (c) and allelic ratio (d).

To further evaluate the cytotoxic properties of AKN-028, and to compare it to the specific FLT3-inhibitor AC220, both compounds were analyzed in 11 primary AML samples using the FMCA. Selection of patient samples was based solely on sample availability and all drugs were run in parallel. As previously, AKN-028 achieved a full dose-dependent response in all samples. For AC220, most patient samples had a dose-dependent effect to a certain degree, and then reached a plateau, sometimes with a cell survival of >50%. This is in accordance with data published previously[116].

As we were searching for a compound able to achieve full efficacy, we chose to calculate IC_{50} values only for curves with stable upper and lower plateaus ranging approximately between 0 – 100 % survival index. The IC_{50} was determined from log concentration–effect curves in Graph Pad Prism (GraphPad software Inc., CA, USA) using non-linear regression analysis and subsequently presented on a log x axis. Since the restraints for the IC_{50}, as well as the projection of the curves, varies between our study and previously published data for AC220[116], the results cannot be directly compared.

Previous reports indicate that the use of FLT3 inhibitors might be more rewarding in the relapse situation compared to up front at diagnosis[155,156]. In our material, there was a trend towards greater efficacy of AC220 in relapse patients (the most sensitive group (Unique patient number (UPN) 16,17,18) contained 3 relapses, the intermediate group (UPN 14, 19, 20, 21) 1 newly diagnosed and 3 relapses, the least sensitive group (UPN 15, 22, 23, 24) 3

newly diagnosed and 1 relapse, but the material is obviously too small to draw any definite conclusion. As for AKN-028, no significant difference was seen between newly diagnosed and relapsed patients (Students t-test p = 0.10).

Sequence-dependent improvement of antileukemic activity when AKN-028 is combined with standard chemotherapeutic agents

To identify possible drug synergies, especially since the effect of FLT3 inhibitors used as single agents has been reported as limited[154], AKN-028 was tested in combination with the conventional cytotoxic agents cytarabine and daunorubicin, both frequently used in AML. Studies using the FMCA revealed a sequence dependent synergy with better antileukemic activity when MV4-11 cells were exposed to standard chemotherapy simultaneously or for 24 hours before adding AKN-028, whereas antagonism was observed when cells were pre-treated with AKN-028. Similar results have been reported previously and the sequence dependent antagonism has been attributed to the induction of cell cycle arrest by certain tyrosine kinase inhibitors[157,158]. Considering that AML is a heterogeneous disease, it is likely that patients will continue to benefit from combination therapies, whether it is combinations of conventional chemotherapy as today, or from a more vertical pathway approach, targeting several members of the same signaling pathway[159]. Whichever strategy one chooses, the dosing sequence is likely to have an impact on the outcome.

In vivo efficacy of AKN-028 in mice

The pharmacokinetic properties of AKN-028 following subcutaneous, intravenous and oral administration were evaluated in C57 black mice. Subcutaneous administration resulted in a short time to peak exposure and a relatively short plasma half-life. Following intravenous administration, a high volume of distribution and high plasma clearance was observed. Oral administration revealed a short time to reach maximum plasma concentration and a good bioavailability of 89%. The results indicate that the anticipated effective plasma exposure could be obtained in mice after oral administration.

AKN-028 was further evaluated in vivo using the hollow fiber mouse model and showed a significant antileukemic effect in both primary AML cells and MV4-11 in the animals.

In conclusion, AKN-028 is a novel TKI with activity in AML, irrespective of FLT3 status and displaying a sequence dependent in vitro synergy with standard antileukemic agents. Good oral bioavailability and cytotoxic effect in vivo have made AKN-028 a candidate drug for clinical studies (ongoing, ClinicalTrials.gov NCT01573247).

Characterization of the novel tyrosine kinase inhibitor AKN-028 in AML (Paper IV)

AKN-028 induces cell cycle arrest in MV4-11 cell line

The aim of Paper IV was to further characterize the actions of the new tyrosine kinase inhibitor AKN-028 (presented in Paper III). AKN-028 is a potent FLT3 inhibitor and displays in vitro anti-leukemic activity in a wide range of AML patients, irrespective of FLT3 mutation status or the quantitative expression of FLT3. The previously reported sequence dependent effects when AKN-028 is combined with standard chemotherapeutic agents, a pattern which in other TKIs have been linked to the induction of cell cycle arrest, led us to perform cell cycle distribution analysis. After 72 hours of exposure to AKN-028 or positive control ATRA, cell cycle distribution was analyzed by flow cytometry, revealing a dose dependent G_0/G_1 arrest after AKN-028 treatment.

AKN-028 induces significant changes in gene expression that differs from midostaurin

Using microarray technique, the global changes in gene expression after drug exposure to AKN-028 or to reference kinase inhibitor midostaurin were studied in two AML cell lines and leukemic cells from one AML patient. In the subsequent analysis, we searched for changes in expression of transcript levels after AKN-028 treatment, compared to vehicle control treated cells, the changes had to be consistent in all three cell types tested. With these criteria and the demand of >twofold difference, mean signal >5 and adjusted $p<0.05$, treatment with AKN-028 resulted in significantly altered gene expression in over 700 genes, 430 transcripts were downregulated and 280 were upregulated. By contrast, treatment with midostaurin caused considerably fewer alterations of transcript levels compared to vehicle treated cells. Principal component analysis (PCA) was used to visualize the data, see Figure 6.

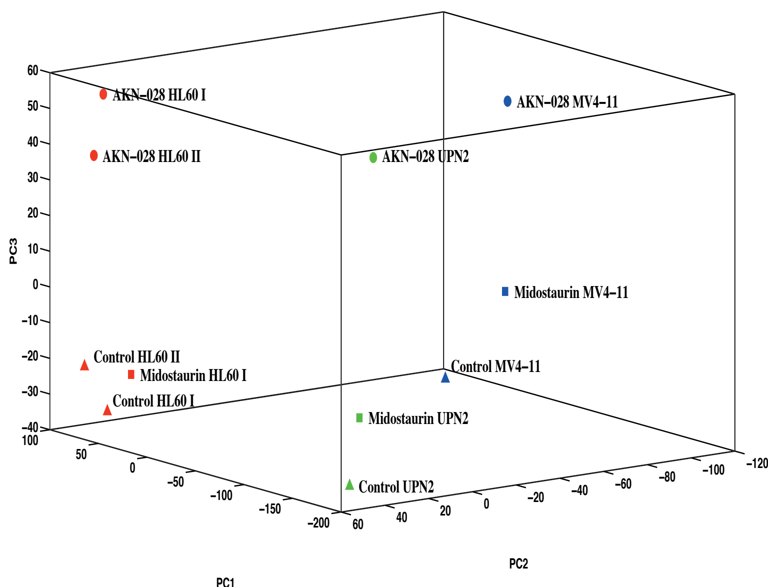

Figure 6. Principal component analysis of results from the gene expression study.

Gene expression analysis produces a substantial amount of data and to analyze these, we performed a gene set enrichment analysis (GSEA). Average fold change of all three cell types tested was used as rank metric for AKN-028-treated samples versus control, and for midostaurin-treated cells fold change versus control. In this way, the most upregulated genes will be found in the top of the rank list, whereas the downregulated ones will end up in the bottom. GSEA utilizes predefined gene sets to determine whether these genes are enriched at the extremes of the ranked list. Our analysis revealed enrichment of genes associated with the proto-oncogene c-myc among the downregulated ones in both AKN-028 and midostaurin treated cells, possibly suggesting a class effect for certain kinase inhibitors.

Myc plays an important role in the regulation of cell proliferation, differentiation and apoptosis. Deregulation of Myc is linked to many types of cancer and it is also the most frequently amplified oncogene[160,161]. In cancer, elevated Myc levels can give rise to a wide variety of changes, affecting for example chromatin structure, metabolic pathways, cell adhesion, apoptosis and cell size[162]. A recent study identifies Myc as a DNA-binding transcription factor with a possible role as inducer of transcript amplification of the existing gene expression program in the malignant cells[162]. With its broad range of actions, Myc in cancer is not likely just a one-dimensional pathway of strictly sequential events. Like for most signal transduction, a three-

dimensional view including multiple intricate interconnected circuits might be more accurate.

Although the pattern for the downregulated genes seems to have similarities between AKN-028 and midostaurin-treated cells, the pattern for the upregulated ones appear to be more AKN-028 specific. Our analysis indicates an enrichment of histone genes as well as ribosomal genes, but the actual impact of these findings remains to be elucidated.

Kinase activity in AML cells is inhibited by AKN-028 in a dose-dependent manner

The tyrosine kinase activity profile was investigated in two AML cell lines and three primary AML samples; cells represented varying sensitivity to AKN-028 in vitro. Our results suggest that AML cells relatively sensitive to AKN-028 in vitro have a higher basal peptide phosphorylation level than more resistant ones. Kinase activity was inhibited by AKN-028 in a dose dependent manner in all samples tested, reaching approximately the same level. These results indicate that the variation in cytotoxic activity of AKN-028 in vitro may be due to the difference in overall kinase expression between different AML cells. Among the peptides with the highest overall phosphorylation signal and the ones most inhibited by AKN-028, we found several peptides linked to different malignancies, for instance Src. Summarizing all findings, we hypothesize that AKN-028, by inhibition of FLT3, reduces Src-activity and subsequently reduces Myc activity, in turn inducing cell cycle arrest and/or apoptosis. As always with hypothesis, as well as with hits from this type of profiling assays, they need to be confirmed by detection of actual target inhibition.

A surprising and interesting finding in the tyrosine kinase activity profiling is the discrepancy in overall kinase activity in different AML samples. If verified in larger sets of patient samples, tyrosine kinase profiles could add information in the classification of AML cases, not just as a possible predictive biomarker, but also on a more basic biological and pathological level.

Conclusions and future perspectives

The results of this thesis may be summarized as follows:

- The combination of high-throughput screens, direct target-compound interaction assays and sequential cellular screens including both cell lines and primary patient samples can be a successful approach in identifying new targeted drugs.
- Using this strategy, we have identified the previously known EGFR-inhibitor gefitinib and the two novel tyrosine kinase inhibitors AKN-032 and AKN-028 as compounds with clear in vitro cytotoxic activity in AML.
- AKN-028 has potent cytotoxic activity in AML samples, irrespective of FLT3 mutation status or quantitative FLT3 expression, and displays a sequence dependent synergy when combined with standard chemotherapeutic agents cytarabine or daunorubicin. Good oral bioavailability and cytotoxic effect in vivo has made AKN-028 a candidate drug for clinical studies (ongoing).
- Treatment with AKN-028 induces a significantly altered gene expression in AML cells with an enrichment of the Myc pathway among the down-regulated genes.
- Tyrosine kinase profiling of AML cell lines and primary AML samples show a dose dependent inhibition of kinase activity after in vitro exposure to AKN-028.
- Tyrosine kinase profiling reveals a difference in overall kinase activity between different AML samples. If verified in larger sets of patient samples, tyrosine kinase profiles could add information in the classification of AML cases, not just as a possible predictive biomarker, but also on a more basic biological and pathological level.

During the course of this thesis, the novel tyrosine kinase inhibitor AKN-028 has moved from an early promising screening hit compound into a clinical trial. Drug discovery is a lengthy process with multiple steps and milestones. The properties and effects of a hit and eventually a lead compound must be examined by a variety of methods and assays. The practical execution and the following interpretation of the results require multifaceted expertise, emphasizing the need of collaboration between people with different skills and background. As academics and clinical hematologists, we can contribute

with the knowledge of and access to the ultimate contemplated beneficiary of the process: the patient.

AML is a very heterogeneous disease, relatively responsive to initial chemotherapy but unfortunately prone to relapse. Against this background, the “one-solution-for-all” treatment plan available today is not particularly attractive and calls for improvement. With AML being such an aggressive disease, it is likely that patients will continue to benefit from a combination of drugs. Thus, finding the role for new signal transduction modulators in the treatment regimens, and how best to combine them with the conventional chemotherapeutics used today, is an important challenge. Furthermore, the new approach of vertical combinations, hitting several targets in closely related pathways, is intellectually appealing and needs to be explored further both in vitro and in vivo. However, whether one combines new agents with conventional chemotherapy or with other targeted therapies, the sequence in which this is done will most likely have an impact on the results.

AKN-028 is currently under investigation in an international two-part multicenter phase I/II study. In parallel, we would like to expand the previous kinase activity profiling studies by examining the AML samples in a serine/threonine tyrosine kinase array. With the obtained kinase activity profiles, we plan to proceed to a pathway analysis, aiming to further elucidate the mechanism of action of AKN-028 in AML. Potential hits/targets, like for instance the Src-family kinases in the performed study, need to be confirmed by measurement of actual target inhibition in AML cells, using for example western blot analysis or equivalent methods.

Better understanding of the disease biology is a key element when it comes to improving the outcome for AML patients. The field of genetics is rapidly expanding, as is the knowledge of signal transduction in malignancies and new targeted therapies. Linking all this information is essential in directing the right therapy to the right patient, and the role of personalized medicine is likely to expand. Finding predictors of drug response is an important part of this process. The data from the tyrosine kinase activity profiling in Paper IV is particularly interesting in this aspect as it could provide a new classification tool for AML patients. Naturally, the hypothesis of difference in overall kinase activity needs to be examined in a larger set of patients. However, an approach linking results from tyrosine kinase activity profiling to clinical data, genetic information and in vitro cytotoxicity profiles, could be a step in achieving a more individualized treatment plan for AML patients. Thus, we plan to use the tyrosine kinase peptide microarray system to profile a larger number of primary AML samples. The obtained kinase activation profiles will then be correlated to clinical information, cytogenetic properties and in vitro cytotoxicity data for several potential antileukemic agents; cytotoxicity data will be acquired by use of the FMCA.

Svensk populärvetenskaplig sammanfattning

Varje år insjuknar cirka 330 vuxna svenskar i akut myeloisk leukemi (AML). AML är en livshotande, malign sjukdom som drabbar blodet och kroppens blodbildande organ, benmärgen. Omogna, elakartade celler växer till i benmärgen och tränger undan de normala blodbildande cellerna. Leukemicellerna karaktäriseras av en mängd genetiska förändringar, mutationer, vilka bl.a. drabbar receptorer. Dessa molekyler, som ofta återfinns på cellytan, skickar vid aktivering signaler till cellen för att initiera t.ex. celldelning, utmognad eller programmerad celldöd. En av de vanligaste mutationerna inom AML drabbar en särskild receptor som heter FLT3, vilken tillhör gruppen tyrosinkinaser.

Dagens leukemibehandling baseras på kombinationer av cellgifter, cytostatika. Cellgifter skadar tumörceller och hindrar dem från att dela sig men påverkar tyvärr också många friska celler, vilket kan leda till biverkningar. Trots mycket intensiv behandling med cytostatika drabbas många AML-patienter av återfall och prognosen är på sikt dyster. Gruppen blodcancersjukdomar, där AML ingår, utgör den tredje vanligaste cancer-relaterade dödsorsaken i Sverige.

Kunskapen kring de processer som styr cancerutveckling ökar ständigt, och i och med det även ambitionen att skapa nya förbättrade behandlingar. I den här avhandlingen har vi strävat mot att hitta nya, riktade terapier mot AML. Vi har använt en kombination av läkemedelsscreening, en mängd uppföljande in vitro försök (i artificiell miljö) och även in vivo metoder (i hela organismer). Vi har på så vis identifierat tre ämnen som potentiellt aktiva inom AML; gefitinib – en riktad behandling som idag används vid lungcancer, samt två helt nya ämnen – tyrosinkinashämmarna AKN-032 och AKN-028. Dessa tre potentiella läkemedel har undersökts med avseende på antileukemisk effekt men också hur denna utövas i AML-celler från såväl humana tumörcellinjer som primära patientceller.

AKN-028 är en effektiv hämmare av tyrosinkinaset FLT3 och har en kraftig avdödande effekt på celler från AML-patienter, oavsett om FLT3 uttrycks normalt eller är muterat. För att öka effekten och minska risken för resistens ges cancerbehandling som regel i kombinationer och vi har därför testat AKN-028 i kombination med två inom AML vanliga cytostatika, cytarabin och daunorubicin. Försöken visade att AKN-028 ger en synergistisk antileukemisk effekt in vitro i kombination med dessa ämnen, dvs. den sammantagna effekten är större än summan av de ingående delarna. Vidare under-

sökningar har visat att AKN-028 har god effekt på leukemiceller även in vivo och att substansen har ett gott upptag och fördelning i kroppen efter oralt intag. Sammantaget har resultaten gjort att AKN-028 nu undersöks i en internationell klinisk läkemedelsstudie för patienter med AML.

Att klargöra verkningsmekanismen för ett nytt läkemedel är en viktig del av utvecklingsprocessen. Microarray-studier, som mäter förändringar av genuttryck, är en av de metoder som kan användas i detta syfte. Genexpressionsstudier visar att celler från AML-cellinjer eller AML-patienter, som behandlas med AKN-028 uppvisar en kraftig förändring av genuttrycket. Bland de gener som nedregleras mest av AKN-028-behandling återfinns många som associeras med Myc, ett DNA-bindande protein som är en viktig aktör när det gäller t.ex. cellers delning och utmognad. Myc-associerade mutationer har kopplats till en rad cancersjukdomar, däribland AML.

Global profilering av tyrosinkinasaktivitet har tidigare använts för att försöka förbättra förståelsen av signalvägar i leukemi och utgör därmed en värdefull strategi för att identifiera verkningsmekanismen för olika läkemedel. Vi har använt ovan nämnda metod för profilering av ett antal AML-celler, cellinjer såväl som primära AML-celler. Försöken antyder att celler med hög in vitro känslighet mot AKN-028 har en högre basal kinasaktivitet än mer resistenta celler. Samtliga prover visade dock en dosberoende hämning till ungefär samma nivå av aktivitet efter AKN-028-behandling. Resultaten kan indikera att skillnaden i in vitro känslighet mot substansen kan bero på skillnad i basalt kinasuttryck. Om resultaten bekräftas i större patient-material skulle denna typ av profilering kunna ge värdefull information för att rikta nya terapier till rätt patienter, och därmed bidra till att individualisera behandlingen för AML-patienter.

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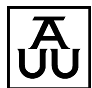

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