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Novel imatinib resistance mechanisms in chronic myeloid leukemia

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1. ABBREVIATIONS

All units of measurement are abbreviated according to the International System of units (SI).

A	Adenosine
ABL	Abelson
ALL	Acute lymphoblastic leukemia
APS	Ammoniumperoxodisulfat
ATP	Adenosine triphosphate
BCR	Breakpoint cluster region
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
CML	Chronic myeloid leukemia
Da	Dasatinib
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	2'-deoxynucleoside-5'-triphosphates
DTT	Dithiothreitol
4E-BP1	4E-binding protein1
EDTA	Ethylene diaminetetraacetic acid
EtBr	Ethidium bromide
FACS	Fluorescence-activated cell sorter
FCS	Fetal calf serum
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GSK-3	Glycogen synthase kinase-3
HEPES	(2-Hydroxyethyl)-1-piperazineethanesulphonic acid
HRP	Horse radish peroxidase
IL-3	Interleukin-3
IL-6	Interleukin-6

IM	Imatinib mesylate (IM, Gleevec®)
IMDM	Iscoves Modified Dulbecco's Medium
kb	Kilobase pair
kD	Kilodalton
MRD	Minimal residual disease
mTor	Mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MTT	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NaCl	Sodium chloride
NI	Nilotinib (AMN107)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Ph^+	Philadelphia chromosome positive
PI_3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PP70S6K	Phosphorylation of p70S6-kinase
RAD001	Everolimus®
RAP	Rapamycin
R-CM	Resistant conditioned media
RNA	Ribonucleic acid
rpm	Retation per minute
RT-PCR	Reverse transcription PCR
SDS	Sodium-dodecyl-sulphate
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA
TKI	Tyrosine kinase inhibitor
Tris	Tris(hydroxymethyl)-amino-methane
UR-CM	Unresistant conditioned media
U	Unit

WM	wortmannin
wt	Wild type

2. INTRODUCTION

In the past decades, much has been learned about the molecular origin of cancer. In particular, the identification of causative oncogenic aberrations led to the rational design of drugs capable of blocking oncogenic signalling. These, so called molecularly targeted therapie have revolutionized cancer therapy. All-trans retinoic acid (ATRA), for example, is used in the treatment of a special subtype of acute myeloid leukemia, acute promyelocytic leukaemia (APL). It can block the oncogenic activity of the underlying chromosomal translocation t (15;17) and transform a formerly poor risk leukemia into a disease with excellent long term prognosis (Longo L. et al., 1990; de The H. et al., 1991; Kakizuka A. et al., 1991; Pandolfi PP. et al., 1992; Fenaux P. et al., 2000). Other examples represent the use of monoclonal antibodies such as trastuzumab to target the oncogenic HER-2 protein in breast cancer (Piccart-Gebhart MJ. et al., 2005; Romond EH. Et al., 2005; Robert N. et al., 2006), rituximab to target CD20 in lymphoma (Coiffier B. et al., 2002; Leahy MF. et al., 2006; Strauss SJ. et al., 2006) and bevacizumab against the oncogenic growth factor, vascular endothelial growth factor (VEGF) in colon cancer (Hurwitz H. et al., 2004; Schulz J. et al., 2005). All these novel substances have been incorporated into the arsenal of conventional tumor therapy, resulting in substantial survival benefits. The most intriguing example for the success of rationally designed molecularly targeted therapy is the development of the specific ABL-tyrosine kinase inhibitor imatinib mesylate (formerly STI571, or CPG57148B, IM, Gleevec^{TM}).

IM led to impressive clinical responses in treatment of Philadelphia-chromosome positive (Ph+) leukemias and has revolutionized the treatment of CML and acute lymphatic leukemia (ALL). However, despite this, therapy resistance, the holy grail of cancer therapy, can not be circumvented by IM. Outright resistance occurs at a frequency of 1-4% annually, but in progressed phases of CML and in Ph+ALL, manifest resistance to IM essentially always emerges after prolonged treatment. Even though most patients with chronic phase of CML achieve a complete cytogenetic remission (CCR), persistence of minimal residual disease occurs in almost all patients. Understanding mechanisms of resistance and persistence to IM and other kinase inhibitors is therefore critical to the issue of potential cure using kinase inhibitors.

2.1 Chronic myeloid leukemia (CML)

2.1.1 History of CML

Chronic myeloid leukaemia (CML) was first described independently by pathologists Bennet, Craigie, and Virchow in 1845(Virchow R.., 1845, Craigie D., 1845, Bennett J., 1845). The Philadelphia (Ph) chromosome as the causative genetic abberration of CML was identified much later in 1960 by Hungerford & Nowell (Nowell PC., Hungerford DA., 1960). They showed a consistent "minute chromosome" abnormality, which they referred to as the Philadelphia (Ph) chromosome. The Ph-chromosome was present in all leukaemia cells from CML, but not from AML patients. In 1973, Janet Rowley's group further confirmed the existence of this chromosome abnormality in CML, but additionally identified that "the minute chromosome" was the result of a reciprocal chromosomal translocation between the long arms of chromosome 9 and 22 (Rowley JD., 1973). These findings were the first to describe that a specific and recurrent chromosomal rearrangement was associated with a specific type of cancer.

2.1.2 Clinical presentation of CML

The natural course of CML is generally characterized by three phases. A benign chronic phase leads over into an accelerated phase and ultimately a terminal blast crisis.

Chronic phase of CML has a consistent, relatively indolent presentation in patients. It is characterized by an expansion of immature and mature myeloid cells and retention of hematopoietic differentiation (Lichtman MA., 1995). Patients often have fatigue, splenomegaly, anemia, and high white blood cell counts in the peripheral blood.

The clinical presentation of accelerated and blast crisis is much more aggressive than chronic phase. Both accelerated and blast phases are characterized by a severe reduction in cellular differentiation, with a replacement of mature cells with immature blasts (Lichtman MA., 1995) and are associated with more severe clinical symptoms including those related to infectious and bleeding complications. Blast crisis resembles acute leukemia which can either be of lymphatic (30%), or more frequently of myeloid character (approx. 70%).

2.1.3 Treatment of CML

CML was previously treated with palliative measures, such as spleenic irradiation or the cytotoxic drug busulfan. Subsequently hydroxyurea, and intensive combination chemotherapy (Kantarjian

HM. et al., 1995) were introduced in CML-therapy and led to a limitedly increased survival. The best conservative treatment with a substantial survival benefit in the last decade was interferon alpha, either alone (Bonifazi F., 2001) or in combination with cytarabine (Kantarjian HM., 1999). With the advent of IM, interferon was no longer the standard therapy of CML (O'Brien SG., 2003).

However, as of today, the only curative treatment remains the allogenetic bone marrow transplantation, which was first applied in the 1980s. Unfortunately, not all patients are eligible for bone marrow transplantation and the cure rate is compromised by a high treatment related toxicity causing a 5-year disease-free survival in transplanted patients as low as 30%-80%, depending on stage of disease, time to transplant, age and donor characteristics (Druker BJ. et al., 2002; Salesse S. et al., 2002).

2.2 BCR/ABL- the molecular cause of CML

2.2.1 The structure of BCR/ABL

As mentioned above, the Ph-chromosome is the unique chromosomal translocation in CML and can be detected in over 95% of CML patients. It is characterized by a reciprocal translocation between the long arms of chromosome 9 and 22 t (9;22)(q34,q11), leading to a fusion of parts of the ABL gene (from chromosome 9) and the BCR gene (from chromosome22). (de Klein A. et al., 1982; Groffen J. et al., 1983; Bartram CR. et al., 1983; Canaani E. et al., 1984; Heisterkamp N. et al., 1985; Shtivelman E. et al., 1985; Mes-Masson AM. et al., 1986; Grosveld G. et al., 1986). The emerging fusion gene BCR/ABL results in a 8.5KB mRNA transcript (Melo JV., 1996), and encodes for a 210 kD chimeric BCR/ABL protein (Ben-Neriah Y. et al., 1986). Sequence analysis disclosed that the BCR/ABL transcript was variable in size and was translated into three isoforms of protein p190, p210 and p230 (Clark SC. et al., 1988; Hermans A. et al., 1987; Mes-Masson AM. et al., 1986; Pane F., 1996) **(Figure 1)**. In human, every of the three isoforms is associated with a distinct type of leukaemia. Type p190 is most often present in B-ALL, p210 is a predominant form of CML, p230 was late found in neutropenic CML (Pane F. et al., 1996).

There are several domains in BCR/ABL contributing its abnormally high ABL kinase activivty compared with ABL alone. An oligomerization motif in BCR/ABL greatly enhances tyrosine kinase activity and cellular transformation (McWhite JR. et al., 1993; Golub TR. et al., 1996), BCR A and B boxes are also required for BCR/ABL activation and transformation ability (Muller AJ. et al., 1991; Pendergast AM. et al., 1991).

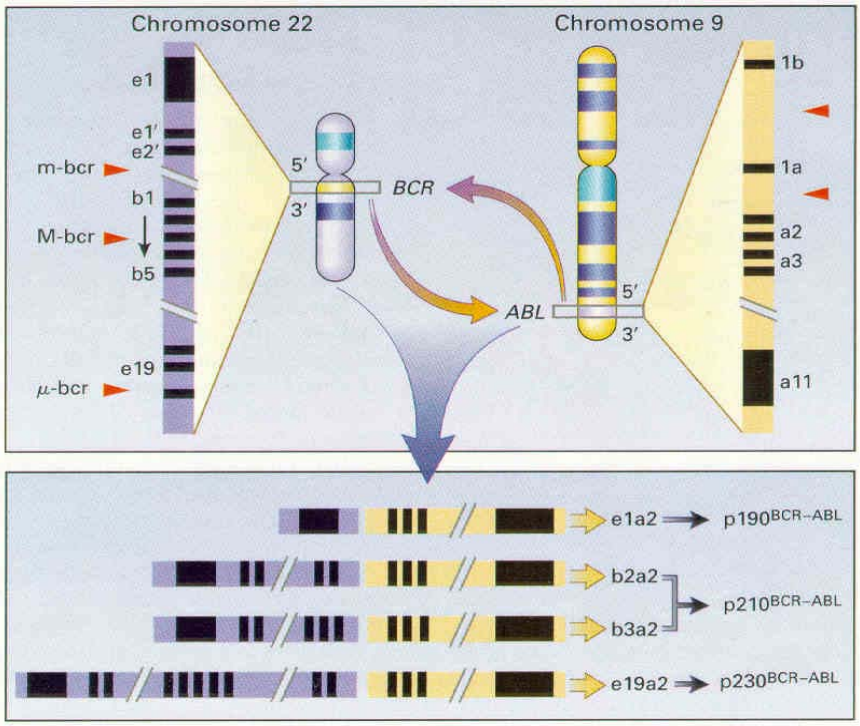

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Figure 1 The Translocation of t(9;22)(q34;q11) in CML and Ph positive ALL.

The Philadelphia-(Ph)chromosome is a shortened chromosome 22 that results from the translocation of 3`(toward the telomere) ABL segments on chromosome 9 to 5` BCR segments on chromosome 22. Various breakpoints (arrowheads) have been identified on chromosome 22 resulting in different fusion messenger RNA moleculaes (e1a2, b2a2, b3a2, and e19a2) of different lengths that are translated into chimeric BCR/ABL protein products of variable size (p190, p210, and p230).

2.2.2 The Signal-transduction pathways affected by BCR/ABL

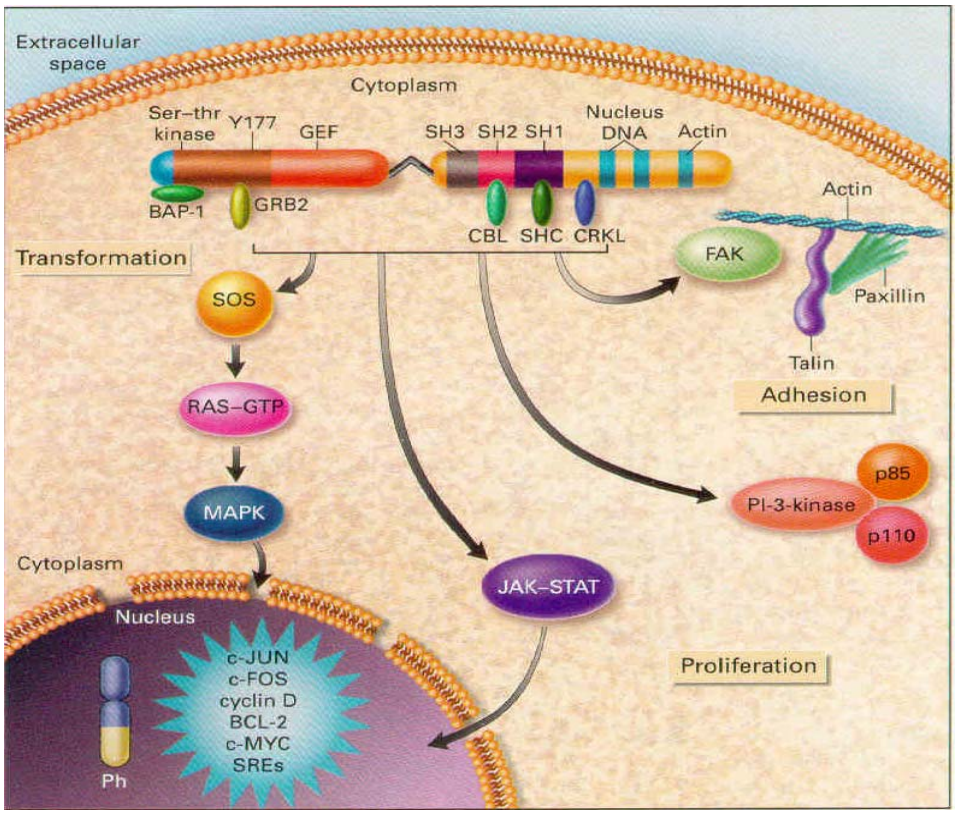
The BCR/ABL fusion protein is a constitutively activated (tyrosine-phosphorylated) non-receptor tyrosine kinase. In its activated state distinct structural domains of ABL within BCR/ABL mediate binding of adapter proteins such as growth factor receptor–bound protein receptor 2(GRB-2), DOK, CRK, CRK-like protein (CRKL), SRC-homology (SHC)-containing proteins, and casitas

B-lineage lymphoma protein (CBL) **(Figure 2)** (Sattler M. et al., 1995; Puil L. et al., 1994; Carpino N. et al., 1997; Oda T. et al., 1994; Ptasznik A. et al., 2002; Andoniou CE. et al., 1996). These interactions cause phosphorylation of adaptor proteins and the activation of various downstream signalling pathways, such as the RAS-MAP-Kinase pathway (Cortez D. et al., 1997; Skorski T. et al., 1997), signalling transducers and activators of transcription (STAT) signalling pathway (Shuai K. et al., 1996), the phosphatidylinositol 3-kinase (PI3K) signalling (Varticovski L. et al., 1991), and MYC-dependent signalling (Stewart MJ. et al., 1995) (Figure 2.2). BCR/ABL also induces autocrine release of cytokines such as interleukin-3(IL-3), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Li S. et al., 2001), which are supposedly also implicated in the biology of CML **(Figure 2)**.

2.2.3 BCR/ABL as a therapeutic target

Targeting BCR/ABL as a therapeutic principle in CML is based on several rationales. First of all, it is a CML specific translocation. 95% of the CML patients harbor the typical chromosomal translocation between the long arms of chromosomes 9 and 22. Secondly, BCR/ABL was shown to be causative for transformation in vitro (Zhao RC. et al., 2001; Era T. et al., 2000) and in vivo (Heisterkamp N. et al., 1990; Daley GQ. et al., 1990). For example, it has been shown that BCR/ABL results in the development of acute leukemia in transgenic mice expressing p185BCR/ABL (Heisterkamp N., 1990). Moreover, several murine transplant models established that BCR/ABL was sufficient not only to cause leukemia, but also T-cell lymphoma (Daley GQ. et al., 1990; Wong S. et al., 2001). BCR/ABL induced leukemias could be transplanted into secondary and tertiary recipients (Gishizky ML. et al., 1993). These results support the notion that BCR/ABL transformed leukemic cells have true cancer stem cell character. Finally, inhibiting ABL was obviously not toxic to cells not expressing BCR/ABL.(Druker BJ. et al., 1996)

The final proof that BCR/ABL is a critical for cellular transformation in any phase of the disease came from the therapeutic efficacy of IM as a specific ABL-inhibitor (Druker BJ. et al., 2001). In 2001, two reports by Druker (Druker BJ. et al., 2001(a); Druker BJ. et al., 2001(b)) documented the clinical efficacy of the BCR/ABL tyrosine kinase inhibitor IM in CML.



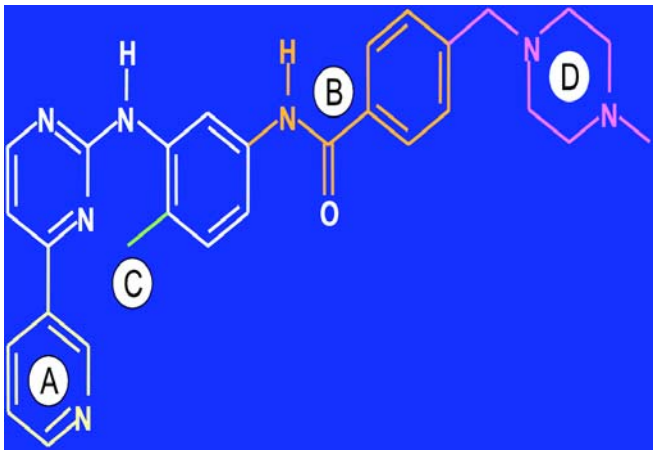
Faderl S., et al.N Engl J Med 1999;341(3):164-172

Figure 2 BCR/ABL-dependent signalling pathways.

2.3 Imatinib mesylate (IM)

2.3.1 Imatinib mesylate, structure and principles of efficacy

The phenylaminopyrimidine molecule CGP57148B **(Figure 3)**, which occupies the kinase pocket of the BCR/ABL protein and blocks access to ATP, thereby prevents phosphorylation of any substate (Goldman JM. et al., 2001) **(Figure 4)**. Preclinical studies showed that the molecule was highly effective in blocking the tyrosine kinase activity of ABL, the stem-cell factor receptor(c-kit), and the platelet-derived growth factor receptor (PDGFR) but had little effect on other tyrosine kinases (Carroll M. et al., 1997; Buchdunger E. et al., 2000; Buchdunger E. et al., 1996). CGP57148 inhibited proliferation of CML cell lines and clonogenic cells from patients with CML but did not affect equivalent control cells (Druker BJ. et al., 1996).



Deininger M., et al. Blood 2005;105:2640-2653

Figure 3 Chemical structure of Imtinib mesylate

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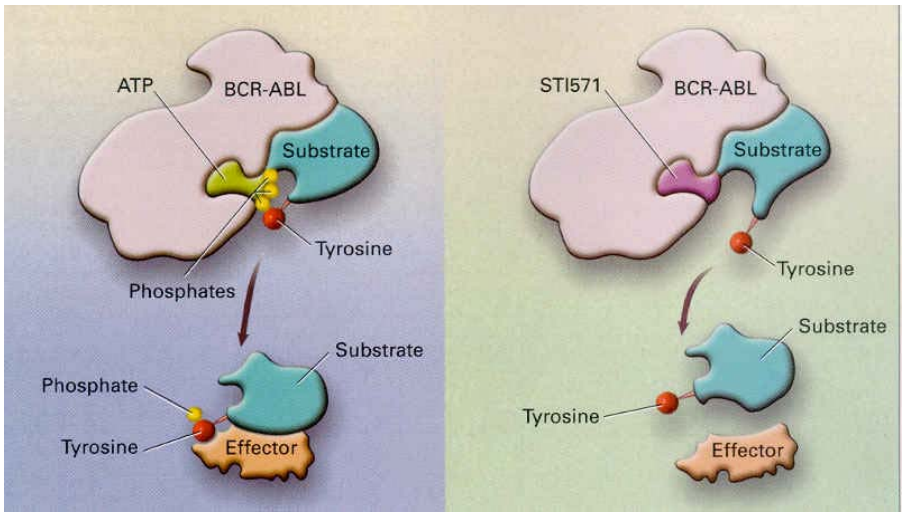
What is the principle of Imatinib binding?

Crystallographic studies elucidated the structural basis of the high efficacy and selectivity of imatinib binding (Shah NP. et al., 2004; Schindler T. et al., 2000). It appears that imatinib binds only to the inactive conformation of BCR/ABL. In this conformation the activation loop closes up the kinase, preventing access of substrates to the kinase and consequently activation of downstream signalling molecules of BCR/ABL. Structural fit of Imatinib to the ATP-binding pocket enables to compete away ATP. Binding of imatinib mainly occurs in the closed (inactive) state, because the activation loop moves outward during activation sterically hindering the access of imatinib to the ATP-binding site when the kinase is active. The structural differences of the ABl-kinase during the closed (inactivated) conformation also explain the specificity of imatinib for ABL and not for other kinases such as for example to SRC-kinases or many others. This ensures a high selectivity of imatinib for BCR/ABL and little side effects in vivo.

2.3.2 The clinical efficacy of imatinib

When added to BCR/ABL positive cell lines, imatinib was found to be highly effective in specifically inhibiting cell proliferation and inducing apoptosis of BCR/ABL positive leukemias while sparing normal hematopoietic cells (Druker BJ. et al., 1996). Subsequent work with this compound confirmed these results (Drucker BJ. et al., 1996; Beran M. et al., 1998; Gambacorti-Passerini C. et al., 1997; Deininger M. et al., 1997) and led to the investigation of this



Goldman JM., et al. N Eng J Med 2001;344(14):1084-6

Figure 4 Likely Mode of Action of STI571.

The left-hand panel shows the BCR/ABL oncoprotein with a molecula of ATP in the kinase pocket. The relevant substrate is phosphorylated on a tyrosine residue and, inits phosphorylated state, can then interact with other downstream effector molecules. When STI571 occupies the kinase pocket (right-hand panel), the action of ATP is inhibited, and the substrate cannot be phosphorylated.

compound in vivo.

2.3.2.1 Imatinib Mesylate treatment leads to remission in most chronic phase CML patients

Phase I studies of therapy-refractory CML patients uncovered the substantial clinical efficacy and very good tolerability of imatinib in vivo (Druker BJ. et al., 2001). The impressive efficacy of imatinib in CML patients provided the final proof for the causative role BCR/ABL in CML. The drug was moved to phase I and II studies in late chronic phase CML patients, resistant or intolerant to IFN alpha (Druker BJ. et al., 2001; Kantarjian HM. et al., 2002). As expected, imatinib was even more effective with most patients achieving complete heamotologic remission (CHR) and a very significant rate of them obtaining major and complete cytogenetic remission (CCR). Consequently, the drug was moved into the first line therapy of chronic phase CML and its efficacy was directly compared with IFNα in a multicenter phase III trial enrolling 1106 patients (O'Brien SG. et al., 2003). This overwhelming and sustained antileukemic efficacy of imatinib in this study led to the introduction of imatinib as the standard therapy in all phases of CML.

2.3.2.2 Failure of imatinib to achieve sustained remission in accelerated and blast phase of CML or

BCR/ABL positive ALL

The efficacy of imatinib was not as good in progressed phases of CML and in BCR/ABL positive ALL as in early chronic phase CML. For example, in a group of 38 CML patients with myeloid blast crisis, only 55% showed response to imatinib and 11% achieved CHR. Only 18% patients kept response over a year because most of the patients relapsed irrespectively of continuous treatment with imatinib (Druker BJ. et al., 2001; Sawyers CL. et al., 2002; Kantarjian H. et al., 2004). Twenty enrolled imatinib-treated patients with lymphoid blast crisis CML and Ph^+ B-ALL had similar response as compared with that of myeloid blast crisis (Druker BJ. et al., 2001) **(Figure 5)**.

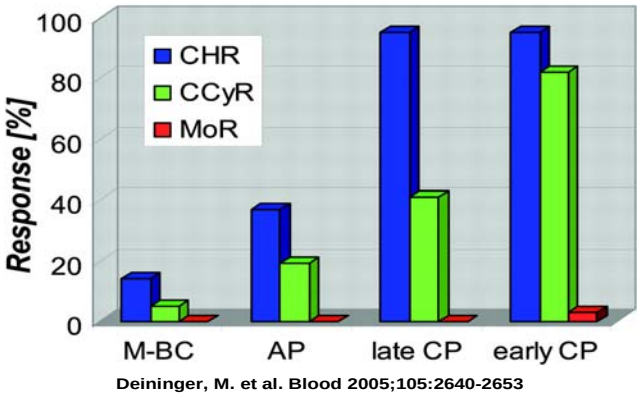


Figure 5 Rates of complete hematologic remission, complete cytogenetic remission, and molecular remission (defined as RT-PCR negativity) in the phase 2 and 3 trials with imatinib monotherapy.

Blue bars indicate complete hematologic remission (CHR); green bars, complete cytogenetic remission (CCR); red bars, molecular remission (MoR). M-BC indicates myeloid blast crisis; and AP, accelerated phase.

2.4 Mechanisms of imatinib resistance

2.4.1 Definition of clinical imatinib resistance and imatinib response

There are two types of imatinib resistances: primary resistance and secondary or "acquired" hematopoietic and cytogenetic resistances according to the criteria recently summarized in a consensus paper of the European Leukemia Net (Baccarani M., 2006). According to this, patients with primary resistance fail to initially respond to imatinib treatment, whereas secondary resistance characterizes loss of a response (hemotologic or cytogenetic). Cytogenetic responses are divided into complete (no Ph+ metaphases), major (less than 35% Ph-positive marrow metaphases) and minor responses (more than 35% Ph+ metaphases). Cytogenetic assessment will be performed every 3 months until obtaining a CCR. After this, cytogenetic assessment will be done once every year to monitor (even in ongoing CCR) for clonal evolution–the emergence of additional Ph-chromsome independent chromosomal aberrations.

After a complete cytogenetic remission has been achieved, molecular remission is monitored via quantitative BCR/ABL-specific PCR. The kinetics and depth of the achievement of a molecular remission could predict the durability of a response, with most patients achieving a 3 log reduction of their initial mRNA value having an almost negligible risk for disease progression (Press RD. et al., 2006).

2.4.2 BCR/ABL–dependent imatinib resistance

If the leukemic clone maintains its BCR/ABL kinase activity in spite of the presence of imatinib, resistance may be considered as BCR/ABL-dependent. Response of CML patients with progressed phases of the disease is always transient, revealed that BCR/ABL activity is reactivated at the time of relapse in most cases (Gorre ME. et al., 2002). The implication of this finding is that despite the numerous secondary genetic alterations that are present in blast phase disease, disease relapse to imatinib most often is conferred by BCR/ABL, and attempts to once again inhibit BCR/ABL activity in these patients holds considerable therapeutic promise.

2.4.2.1 BCR/ABL- overexpression

Approximately 10% of resistant cases are associated with overexpression of BCR/ABL, typically through genomic amplification or the acquisition of additional Ph chromosome (Le Coutre P. et al., 2000; Gorre ME. et al., 2001; Hochhaus A. et al., 2002). Under these circumstances, it is presumed that the intracellular concentration of imatinib is insufficient to inhibit an increased kinase activity of BCR/ABL. Consequently increasing dose would be a rational approach to overcome this type of

imatinib-resistance.

2.4.2.2 BCR/ABL tyrosine kinase domain mutations

BCR/ABL kinase domain mutations represent the most commonly observed mechanism of acquired resistance to imatinib, occurring in 50%-90% of cases (Hochhaus A. et al., 2002; Gorre ME. et al., 2001; Shah NP. et al., 2002; von Bubnoff N. et al., 2002; Branford S. et al., 2002; Al-Ali HK. et al., 2004). To date, more than 40 different mutations have been associated with clinical resistance to imatinib (Gorre ME. et al., 2001; Von Bobnoff N. et al., 2002; Branford S. et al., 2002; Hofmann WK. et al., 2002; Roche-Lestienne C. et al., 2002; Shah NP. et al., 2002; Hochhaus A. et al., 2002). They are located at four distinguishable clusters according to the site of mutations: ATP binding loop (P-loop), activation loop (A-loop) and others**.** The majority of mutations are felt to prevent the kinase domain from adopting the specific conformation to which imatinib bindings (Schindler T. et al., 2000; Shah NP. et al., 2002). Studies have shown that some mutations confer only a moderate degree of resistance, and as a result, dose escalation is predicted to recapture responses in some cases (Von Bobnoff N. et al., 2002; Shah NP. et al., 2002; Corbin AS. et al., 2003), whereas some mutant forms are highly resistant to imatinib, for example, T315I. Mutations in ATP binding loop have been suggested to be associated with poor prognosis (Branford S. et al., 2003; Ian J.Griswold. et al., 2006). However, more recent reports can not confirm this (Jabbour E. et al., 2006). The identification of mutations as a major mechanism of imatinib resistance has been the rationale to develop novel kinase inhibitors that could bind very efficiently to mutated BCR/ABL. Nilotinib and Dasatinib belong to this new class of second generation inhibitors capable of blocking both wild type and almost all mutated ABL (Weisberg E. et al., 2005; Shah NP. et al., 2004).

2.4.3 BCR/ABL-independent imatinib resistance

In vitro evidence suggested the existence of BCR/ABL independent types of imatinib-resistance, BCR/ABL positive cell lines survived in presence of imatinib although the BCR/ABL kinase activity was potently inhibited. Mechanisms of BCR/ABL independent resistance include the activation of alternative anti-apoptotic pathways such as the SRC-signalling pathway. (Donato NJ. et al., 2003) Alternatively, imatinib-resistance LAMA84 cells showed increased expression of MDR2, an efflux pump that decreases the intracellular concentration of imatinib (Mahon FX. et al.,

2000). NF-κB was also shown to be involved in BCR/ABL-independent imatinib resistance in several cell lines (Dai Y. et al., 2004). In primary cells it has been shown that solely blocking the SRC kinase lyn is sufficient to induce apoptosis in BCR/ABL positive primary cells (Ptasznik A. et al., 2004). Together, BCR/ABL remains the primary target in imatinib-resistance disease, but BCR/ABL- independent "off-target" inhibition appears to contribute to overcome imatinib-resistance.

2.4.4 Strategies to overcome imatinib resistance

Identifying molecular mechanisms of imatinib resistance was a prerequisite to develop strategies to overcome it. Several strategies are used to overcome clinical imatinib-resistance:

1. Dose escalation of imatinib

Retrospective data suggest that dose escalation (600 to 800mg/day) can overcome hematologic or cytogenetic resistance in some patients (Kantarjian HM. et al., 2003), although these responses may not be maintained (Marin D. et al., 2003). An important consideration is the specific type of mutation. Dose escalation is likely to be effective in the case of mutations with a low or moderate level of resistance to imatinib, such as H386P, but not in highly resistant mutants such as T315I or E255K (Corbin AS. et al., 2003).

2. Combinations with newer signal transduction inhibitors

Many drugs have been tested for their synergism with imatinib (La Rosee P. et al., 2002) such as cytarabine or homoharringtonine. However, newer signal transduction inhibitors such as Ras signaling inhibitors (Daley GQ. et al., 2003), RAD001 (Dengler J. et al., 2005), histone deacetylase inhibitors (Yu C. et al., 2003) and multi-kinase inhibitors (SKI606) (Golas JM. et al., 2003; Golas JM. et al., 2005) or non ATP-competitive inhibitors (Gumireddy K. et al., 2005) are also currently tested in clinical trials to overcome poor molecular responses and manifest imatinib resistance.

3. Second generation ABL- inhibitors

Several compounds with inhibitory activity toward the ABL kinase have been developed. Nilotinib (AMN107) is a very strong and more selective inhibitor of the ABL kinase with approximately 25-fold increased potency relative to imatinib (Weisberg E. et al., 2005; O'Hare T. et al., 2005). Nilotinib has recently shown significant activity in a phase I clinical trial in patients resistant or intolerant to imatinib (Kantarjian HM. et al., 2006). BMS354825 (dasatinib, Sprycel®) belongs to a second family of very effective dual kinase inhibitors. It blocks the ABL and SRC kinase family and has also been proven effective in a clinical phase I study (Talpaz M. et al., 2006) and several

ongoing phase II studies. The overall cytogenetic remission rate was about 40 to 50% in imatinib-resistant CML patients in chronic phase; 40% in accelerated phase; and approximately 20% in blast crisis and Ph positive ALL.

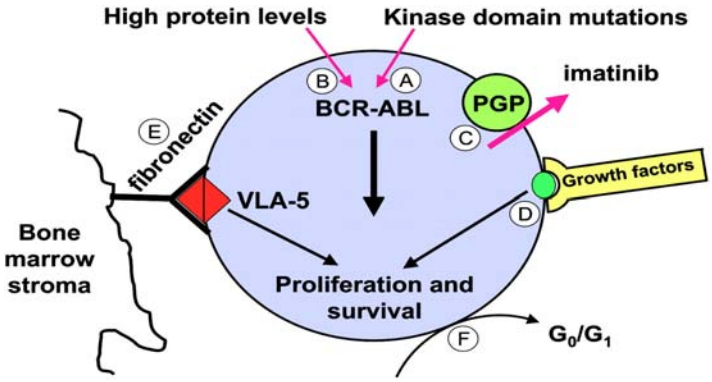
2.5 Persistance

Despite the fact that imatinib is very efficacious in treating BCR/ABL-positive leukaemia with most patients achieving a CCR, minimal residual disease is almost always detectable and referred to as disease persistence (Hughes TP. et al., 2003; Bhatia R. et al., 2003). Disease persistence is of considerable clinical significance, because it indicates that imatinib alone is not capable of eradicating CML. Accordingly, pausing imatinib usually invariably causes a relapse of the disease. So far mechanisms of disease persistence are largely unclear. However, it is well accepted that persisting CML and ALL cells are quiescent (Holyoake T. et al., 2003; Holtz MS., 2005), overexpress BCR/ABL (Jiang X. et al., 2003; Copland M. et al., 2006) and efflux pumps which maintain low intracellular levels of imatinib (Jordanides NE. et al., 2006). It is therefore believed that imatinib may prevent stem cells proliferation, but be unable to eliminate them. Overall mechanisms are summarized in **(Figure 6)**.

Other mechanisms may also contribute to CML persistence. For example, when BCR/ABL was inhibited by imatinib in primary CD34+ CML progenitors, a counterintuitive, cytokine dependent activation of the mitogen-activated protein kinase (MAPK) occurred (Chu S. et al., 2003). Moreover, adhesion to integrins also inhibited apoptosis of BCR/ABL$^+$ cell lines (Bueno-da-Silva AE. et al., 2003). Kinase mutation may also mediate persistence (Chu S. et al., 2005). Identifying mechanisms of disease persistence will be of decisive importance to the issue of cure using molecular inhibitors.

2.6 Aim of the Project

Imatinib has become the standard therapy for patients with BCR/ABL-positive leukemias. Unfortunately, even after years of treatment with the drug, neither CML nor Ph+ALL patients can be cured with imatinib alone. In fact, imatinib resistance regularly occurs when imatinib is given to patients in progressed phases of CML and Ph+ ALL. The main mechanism of resistance is the occurance of mutations in the BCR/ABL kinase domain. New inhibitors which potently block almost all known kinase mutations have therefore been developed and recently also introduced into



Deininger, M. et al. Blood 2005;105:2640-2653

Figure 6 Potential mechanisms underlying disease persistence (molecular refractoriness**).**

(A) Kinase domain mutations that confer moderate resistance to imatinib.(B) BCR-ABL levels may be particularly high in the most primitive leukemic stem cells.(C) Inadequate intracellular levels of imatinib as a result of PGP expression.(D) Physiologic growth factor signalling or (E) integrin signals may maintain viability even with BCR-ABL kinase activity completely inhibited. (F) Quiescent (dormant) cells may be protected against imatinib. VLA-5 indicates very late activation antigen-5.

clinical trials for the treatment of imatinib resistance disease. However, in vitro evidence and an increasing body of in vivo evidence suggest that even these new compounds can not achieve cure. To eradicate minimal residual disease and to target mutation independent mechanisms of imatinib resistance remain therefore of significant clinical value.

To identify mechanisms of disease persistence and resistance we established a longitudinal imatinib-resistance induction model using clonal populations of BCR/ABL-positive LAMA84 cells. In these experiments, many individual subclones of LAMA84 cells, were exposed in parallel to increasing concentrations of imatinib and after intervals aliquots of the cells, protein lysates, and RNA were frozen for later analysis. Primarily, we sought to identify very early biologic changes that mediate incipient imatinib-resistance.

In a second set of experiments and based also on the long known mechanism of autocrine

stimulation in BCR/ABL positive leukemias, we asked, whether autocrine pathways may also contribute to imatinib resistance development.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and equipment

Chemicals

Acrylamide/Bis-acrylamide	Roth, Karlsruhe
Agarose	Gibco-BRL, Neu Isenburg
Ammoniumperoxodisulfat (APS)	Sigma-Aldrich, Seelze
AMN107-NX (free base)	NovartisPharma(Basel, Switzerland)
Aqua dest	B. Braun Melsungen AG
Bovine serum albumin (BSA)	Sigma-Aldrich, Seelze
Bromophenol blue sodium salt	Serva, Heidelberg
Calcium chloride	Merck, Darmstadt
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
1,4-Dithiothreitol	Roth, Karlsruhe
Ethanol	Merck, Darmstadt
Ethidiumbromide	Roth, Karlsruhe
Ethylene diaminetetraacetic acid disodium salt (EDTA)	Merck, Darmstadt
Ficoll-Pague™ PLUS	Amersham Biosciences
Glycerol	Merck, Darmstadt
Glycine	Sigma-Aldrich, Seelze
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	Roth, Karlsruhe
Igepal CA-630 (indistinguishable from NP-40)	Sigma-Aldrich, Seelze
Imatinib mesylate	NovartisPharma(Basel, Switzerland)
Leupeptin	Sigma-Aldrich, Seelze
Methanol	Merck, Darmstadt
ß-Mercaptoethanol	Merck-Schuchardt, München
Natriumdesoxycholat	Sigma, St Louis, MO, USA
Orthovanadate	Sigma, St Louis, MO, USA
Pepstatin	Sigma, St Louis, MO, USA
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, Seelze
Propidium iodide(PI)	Sigma, St Louis, MO, USA
RAD001 (Everolimus®)	NovartisPharma(Basel, Switzerland)
Rapamycin	Sigma-Aldrich, Seelze
SH-6	Alexis
Sodium chloride	Sigma-Aldrich, Seelze
Sodium dodecyl sulfate (SDS)	Merck, Darmstadt
N,N,N´,N´-Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe
Tris(hydroxymethyl)aminomethane (Tris)	Roth, Karlsruhe

Tween®20	Roth, Karlsruhe
Wortmannin(WM)	Calbiochem Bad Soden

Cell Culture Media and Antibiotics

FCS, trypsin, glutamine	Gibco-BRL, Karlsruhe
IMDM medium (SH30228.01) HyClone®	HyClone
Metho Cult™ H4230(no Cytokines)	Stem Cell Technologies
rhMIP-1α	Calbiochem Bad Soden
rhSCF	Pepro Tech, London
rhG-CSF	Pepro Tech, London
rhGM-CSF	R&D systems
rhIL-3	Pepro Tech, London
rhIL-6	Pepro Tech, London
rhIL8	Calbiochem Bad Soden
rhOncostatin M	Calbiochem Bad Soden
RPMI 1640 medium (72400-021)	Gibco-BRL, Karlsruhe
Penicillin/Streptomycin (15140-114)	Biochrom KG, Berlin, Germany

Equipment

Agarose gel electrophoresis chambers	BioRad, München
Amaxa nucleofector device	Amaxa GmbH, Cologne, Germany
Cell culture incubator	HERAEUS, Germany
Clean bench	Kendro, Hanau
ELISA reader	Labsystems Multiskan RC
Freezer	HERAEUS, Germany
GeneAmp® PCR system 9600	Applied Bioystems, Darmstadt
PCR amplifying machine with gradient cycler	Eppendorf AG
Inverted microscope (DMIL)	Leica, Wetzlar
OPTIMAX® film processor	PROTEC processor-Technology
Power supply units (MODE/250EX)	Gibco BRL electrophoresis Power supply
Semi-dry blot apparatus	BioRad, München
Sequence detection system (ABI PRISM 7700)	PE Applied-Biosystems
Superspeed refrigerated centrifuge	Eppendorf AG
X-ray film processor (UVT 2035)	Herolab

Miscellaneous

BioMax-MR films	Kodak, Rochester, New York, USA
DNA and protein size markers	Roche, Mannheim
Gel-blotting-paper(GB003)	Schleicher&Schuell BioScience GmbH
Nitrocellulose transfer membrane	Schleicher&Schuell BioScience GmbH
Sterile plastic ware for cell culture	Greiner Bio-one GmbH, Solingen, Germany

3.1.2 Cell lines

LAMA-84

BCR/ABL-positive LAMA-84 cells (LAMA) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany).

Morphology: single, round to polymorph cells growing in suspension, some cells are loosely adherent.

Properties: $CD3^-$, $CD4^-$, $CD11b^+$, $CD13^+$, $CD14^-$, $CD15^-$, $CD19^-$, $CD33^+$, $CD34^-$, HLA-DR(+), $CD41^-$, $CD42^-$.

Culture: 90% RPMI 1640 + 10% FBS maintain at 0.5-1.0 × 10^6 cells/ml;optimal sptimal split ratio of 1:2 to 1:3 every 2-4 days; dislodge any adhering cells by shaking cultue flask at 37°C with 5% CO_2 saturation density at about 2.0 ×10^6 cells/ml; doubling time of ca. 50 hours.

BA/F3

Origin: IL-3 dependent murine pro B cell line established from peripheral blood; apparently derived from BALB/c mouse.

Morphology: mostly single, round (some polymorph) cells in suspension (or occasionally in clumps)

Culture: 90% RPMI 1640 + 10% FBS + 10 ng/ml IL-3. Ectropic expression of BCR/ABL could enable BA/F3 to grow independently on IL-3.

3.1.3 Buffers and solutions

10%APS: 1g ammoniumpersulfat in 10ml destilled water.

1×binding buffer for apoptosis detection: 10ml HEPES pH7.4, 140mM NaCl, 2,5mM $CaCl_2$.

Blotting buffer: 25mM Tris-OH, 114mM Glycine, 10% Methanol.

10×Electrophorese buffer: 250mM Tris-OH, 1.9M Glycine, 2.5%SDS.

MACS buffer: 2μM EDTA, 0.5%BSA in PBS.

2×PP (for IP): 2.5 ml 1.5M Tris pH8.8, 3ml 20% SDS, 2ml glycerol, 0.25ml 1% bromophenol blue and 1ml β–Mercaptoethanol.

4×PP: 60mM Tris-HCl pH6.8, 25% glycerol, 2% SDS, 0.7M β–Mercaptoethanol, 0.1% bromophenol blue.

10× Red blood cell lysis buffer: Dissolved 8.02g Ammoniumchlorid, 1g Kaliumhydrogencarbonat, 0.037g EDTA with H_2O to 100ml.

RIPA: 20mM Tris PH7.4, 150mM NaCl, 5mM EDTA, 1%SDS, 0.5% NaDeoxycholat with fresh 1mM PMSF, 2µg/ml Leupeptin, 4µg/ml Aprotinin, 1.5µg/ml Pepstatin, 1µg/ml Trypsininhibitor and 50mM NaF.

Strip-buffer: 0.1M β–Mercaptoethanol, 2% SDS and 62.5mM Tris HCL (pH 6.7).

1×TAE (pH 8.0) buffer: 40mM Tris-acetate/1mM EDTA.

10× TBS buffer: 200mM Tris-OH pH 7.5, 1.37M NaCl.

1× TTBS buffer: 1L 1×TBS and 1ml Tween 20.

3.1.4 Antibodies and Kits

Antibodies

Anti-c-ABL antibody (sc-23)	Santa Cruz Biotechnology,Inc.,Heidelberg,Germany
Anti-AKT	Cell Signaling Technologies, Berverly, MA
Anti-ß-Actin(AC-74)	Sigma-Aldrich, Seelz
Anti-hGM-CSF neutralizing antibody	R&D systems
Anti-human-CD34 PE34 PE-Cy7	BD Biosciences, Heidelberg, Germany
Anti-mouse IgG/HRP	DAKO A/S. Danmark
Anti-phospho-AKT	Cell Signaling Technologies, Berverly, MA
Anti-phospho-JAK2	Cell Signaling Technologies, Berverly, MA

Anti-phospho-Crkl (Tyr207)	Cell Signaling Technologies, Berverly, MA
Anti-phospho-p^{T389} p70S6K(Ser389)	Cell Signaling Technologies, Berverly, MA
Anti-phosphotyrosine, clone 4G10	Upstate. USA
Anti-Rabbit IgG/HRP	DAKO A/S. Danmark
Biotinylated mouse anti-human GM-CSF(CD116)	BD Biosciences, Heidelberg, Germany
Streptavidin-APC	BD Biosciences, Heidelberg, Germany
FITC lebelled goat anti-rabbit IgG	BD Biosciences, Heidelberg, Germany
PE conjugated mouse anti-stat5 (pY694)	BD Biosciences, Heidelberg, Germany
Kits	
BCA™ protein Assay kit	PIERCE Rockford, USA
AKT Kinase Assay Kit	Cell Signaling Technologies, Berverly, MA
AnnexinV-FITC apoptosis detection Kit	Alexis
Cell growth determination kit, MTT based	Sigma-Aldrich, Seelz
CellTiter 96 Aqueous One Solution Reagent (MTS KIT)	Promega, Madison, WI
Cell-line specific Nucleofector Kit V	Amaxa GmbH, Cologne, Germany
Human Cytokine Antibody Array C Series 1000	Ray Biotech, Inc.
Human GM-CSF ELISA Kit	Diaclone BESANCON Cedex, France
QuantiTect™ SYBR® Green PCR kit	Qiagen Hilden

3.1.5 Oligonucleotides

Human AKT-1 specific siRNA (high performance validated siRNA) Qiagen Hilden, Germany.

All primers were supplied by MWG-Biotech, Ebersberg, Germany.

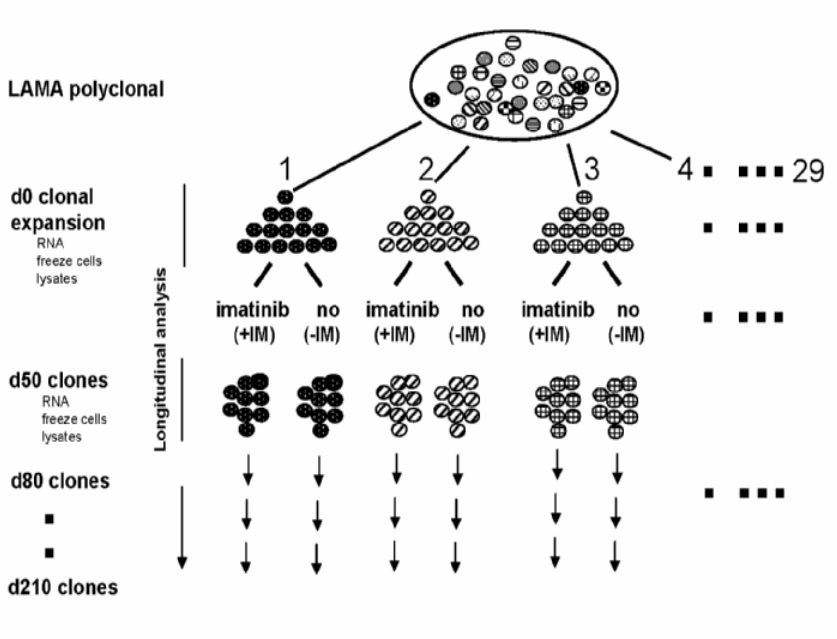
3.1.6 Analysing softwares

CellQuest and ModFit software	Becton Dickinson, San Jose, CA,
FlowJo software	Tree Star Inc., Oreagon, USA
Sequence Detector v1.6 software	PE Biosystems

3.2 Methods

3.2.1 Cell culture and clonal in vitro resistance induction

Cell line: BCR/ABL positive LAMA-84 cell line (LAMA) was cultured in RPMI1640 medium supplemented with 1% glutamine, 10% fetal calf serum (FCS), 1% penicillin/streptomycin. Single cell clones were generated from LAMA-cells by limited dilution. Each clone was expanded to individual cultures. Aliquots of the populations were referred as d0 and cryo-preserved. Each clone was exposed both to rising concentration of IM (+IM) or mock-treatment (-IM, control culture). IM-exposure was started with 0.05 μM and increased every ten days by 0.05 μM only in case of more than 70% viability in culture, as assessed by trypan blue exclusion method. The IM-concentration remained unchanged if the viability was between 30-70%, and IM was withdrawn in case of less than 30% viability, which was referred to as rescue. Rescue periods depended on recovery time. IM was re-added to 50% of the last achieved IM-level in case of > 90% viability in the culture. Cell aliquots, protein lysates and total RNA of control- and IM-exposed cells were cryo-preserved in regular time intervals after d0 (summarized in **Figure 7**). In other experiments, IM-resistance was generated accordingly, but in steady presence of 5nM Rap, which was renewed every three days.



Buchert A., et al. Leukemia 2005; 19:1774-1782

Figure 7 Outline of experimental approach

Clonal Populations of LAMA cells were generated using limited dilution. Clones were expanded to cell line and cultured without IM (-IM, control) or in presence of rising concentrations of IM (+IM), which was increased every ten days only if viability was >70%. Cell aliquots, whole protein lysates and RNA were preserved on d0 and every month thereafter from IM-exposed and -unexposed control cultures of each clone.

Primary cells: 10-20ml peripheral blood or bone marrow samples were obtained after written informed consent. The collection of blood samples was approved by the local ethics committee of the Klinikum, Philipps-Marburg University.

①Isolation of mononuclear cells

Mononuclear cells were separated from 10-20ml peripheral blood or bone marrow samples with Ficoll. Briefly, carefully added blood or bone marrow on the top of Ficoll in the 50ml tube, centrifuged for 20 minutes at 1500 rpm without braking, middle layer cells were carefully taken out and washed with PBS, lysed with 1× red blood cell lysis buffer for 5 minutes to remove contaminated red blood cells, again washed with PBS.

②Enrichment of CD34 positive cells

Mononuclear cells resuspended in MACS buffer (0.2M EDTA, 0,25g/ml BSA.) were mixed with

magnetic beads conjugated mouse anti-human CD34 antibody and incubated on ice for 20 minutes. The mixture was loaded on pre-washed column with MACS buffer, which was entrapped in strong magnetic field. The column was then washed with MACS buffer twice. The cells were harvested by removing the column from the magnetic field, putting syringe core into the column and pushing it quickly and strongly. The collected cells were purified once more with another new column.

CD34 positive cells were cultured in HyQ IMDM medium containing 4mM L-Glutamine, 0.1μM HEPES, 30% FCS, 20ng/ml stem cell factor, 10ng/ml interleukin-6, 20ng/ml granulocyte colony stimulating factor and interleukin-3 (all from PeproTech, London, UK).

3.2.2 Preparation of total RNA from cultured cells

Total RNA was extracted from LAMA cells and primary cells using QIAamp RNA Blood Mini Kit (QIAGEN), according to the manufacturer's instructions. 1-5×10^6 cells were lysed using highly denaturing conditions that immediately inactivate RNases, allowing the isolation of intact RNA. After homogenization of the lysate by a brief centrifugation through a QIAshredder spin column, ethanol was added to adjust binding conditions and the sample was applied to the QIAamp spin column. RNA was bound to the silica-gel membrane during a brief centrifugation step. Contaminants were washed away and total RNA was eluted in 30ul of RNase-free water and stored at – 70°C.

3.2.3 Preparation of protein lysate

1-5×10^6 LAMA84 cells and primary cells were harvested by centrifuging at 1500rpm for 5 minutes at room temperature and rinsed once with cold PBS. The pellet were solubilized in RIPA buffer and incubated on ice for 30 minutes. Cellular debris was removed by centrifuging at 13000rpm for 10 minutes at 4°C. The supernatant was collected and stored at – 70°C.

3.2.4 SDS Polyacrylamide gel electrophoresis

Discontinuous SDS Polyacrylamid gel electrophoresis (Davis, 1964 and Ornstein, 1964) was performed in a vertical system in order to analyze some genes expression in LAMA cells and primary cells. The denatured polypeptides bind SDS and become negatively charged. Because the amount of the SDS bound is usually proportional to the molecular weight of the polypeptide and is independent of its sequence, the mobility of protein-SDS complexes in polyacrylamide gels in

inverse proportional to the size of the protein. By using markers of known size it is therefore possible to estimate the molecular weight of a protein.

SDS polyacrylamide gel electrophoresis was carried out in a discontinuous gel system consisting of an upper stacking gel, a lower resolving gel and an electrophoresis buffer with different PH value and ionic strength than the gel buffers. The samples and the stacking gel contain Tris-CL (PH 6.8), both buffer reservoirs contain Tris-glycine (PH 8.3), and the resolving gel contains Tris-CL (PH 8.8). All components of the system contain 0.1% SDS (Laemmli, 1970). The fast chloride ions in the samples and stacking gel from the leading edge of a moving ion boundary, and the trailing edge is composed of slow glycine molecules. Between both edges of the moving boundary is a zone of lower conductivity and steeper voltage gradient, which sweeps the polypeptides of the sample and deposits them on the surface of the resolving gel. There the higher PH of 8,3 favours the ionization of glycine, so that the charged glycine molecules are moving fast through the stacked polypeptides and travel through the resolving gel immediately behind the chloride ions. Freed from the moving boundary the SDS-polypeptide complexes move through the resolving gel in a zone of uniform voltage and pH and are separated to size by sieving.

For most purposes a 7.5 or 10% resolving gel was prepared. The gel solution was poured into the assembled gel mold between two glass plates separated by 0.75mm think spacers leaving some 1cm space for the stacking gel. The gel surface was overlaid with 100% Ethnol in order to prevent inhibition of polymerization by oxygen. After polymerization was complete (30min) the stacking gel (always 3-4%) was poured on top of the resolving gel, and the comb was inserted.

Samples were prepared in 1×SDS gel-loading buffer by means of a 4×concentrated stock solution. After having added 5% (v/v) ß-Mercaptoethanol or 10%(v/v) 1 M DTT all samples were boiled for 5 min to denature the proteins. After polymerization of stacking gel (30 min) the comb was removed and the gel mounted in the electrophoresis chamber. Both electrode reservoirs were filled with SDS electrophoresis buffer, the wells were cleaned and samples loaded. Electrphoresis was performed at 120 voltage constant power until the bromophenol blue dye had reached the bottom of the gel.

3.2.5 Western blot

Proteins were separated in a SDS polyacrylamide gel and electro-transferred to a nitrocellulose membrane at 10-12 voltage for 1h using a semi-dry blot apparatus according to the instructions provided by the manufacturer. Blocked with 1.5% (v/v) Western blocking reagent (1.5ml Western

blocking reagent in 100ml TBS) for overnight at 4 ^{o}C, the membranes were incubated overnight at 4 ^{o}C respectively with anti-c-ABL (sc-23), anti-Akt, anti-phospho-Akt (Ser 473), anti-phosphorylation form of p70S6 Kinase (Ser389), and anti-phospho-Crkl antibody; alternatively probed at room temperture for 2h respectively with anti-phospho-tyrosine kinase, anti-phospho-stat5 and anti-ß-Actin (AC-74) antibodies. Following incubation, membranes were washed three times with 1×TTBS buffer. HRP-conjugated anti-mouse or anti-rabbit second antibody was applied to membranes for 40minutes' incubation at room temperature. After washed three times in 1×TTBS buffer, the membranes were visualized by incubating with the enhanced chemi-luminescence (ECL) detection reagent (1:1 mixture (v/v) of Reagent 1 and reagent 2) for 1 min and exposing to BioMax-MR films.

3.2.6 Sequencing of the BCR/ABL kinase domain

RT-PCR: 1μg total RNA was reversely transcribed into cDNA in the reaction system containing 1× reaction buffer, 0.5mM dNTP, 10uM hexamer primer, 10units RNase inhibitor, reverse transcriptase 4U for 1 hour at 37°C, followed by stopping reaction with 95°C 5minutes. PCR amplification was performed under following conditions: 1 × reaction buffer, 0.8mM dNTP, 0.5μM of each primer, 0.625 U Taq DNA polymerase in a total volume of 25μl. Primer sequences are showed as below. The cycling conditions were: 94°C 30 sec for denaturation of cDNA template, annealing at 60°C for 30 sec, extension at 72°C for 40 sec, followed by a final cycle of 90°C for 1 minute and 60°C for 10 minutes. The PCR products were visualized by electrophoresis on a 1 or 3% agarose gel and staining with ethidium bromide.

Sequencing: BCR/ABL tyrosine kinase domain [gene accession number: U07563] was sequenced after reverse transcriptase (RT)-PCR amplication from cDNA. Briefly, to amplify the kinase domain, hemi-nested PCR was performed using the following primers: first step, B2B (BCR exon 13) ACAGCATTCCGCTGACCATCAATAAG plus A7 - (ABL exon 7) AGACGTCGGACTTGATGGAGAACT; second step, AN4 + (ABL exon 4) TGGTTCATCATCATTCAACGGTGG plus A7 -. This procedure ensured that the normal, un-rearranged ABL gene was not analyzed. The 675 bp products encoding the BCR-ABL ATP binding pocket and the activation loop were directly sequenced in both directions.

3.2.7 Real time quantitative PCR (TaqMan PCR)

Real-time PCR and RT-PCR are highly sensitive techniques enabling amplification and quantification of a specific nucleic acid sequence with detection of the PCR product in real time. SYBR Green I binds all double-stranded DNA molecules, emitting a fluorescent signal of a defined wavelength on binding. The excitation and emission maxima of SYBR Green I are at 494nm and 521nm, respectively, and are compatible for use with any real-time cycler. The optimisation of the real-time PCR reaction was performed according to the manufacturer's instructions (PE Applied-Biosystems, User Bulletin 2 and the QuantiTect™SYBR® Green PCR Handbooks) but scaled down to 25 µl per reaction. The PCR conditions were standarded according to protocol and all reagents were provided by the kit.

The GAPDH primers were 5'-GAA GGT GAA GTT CGG AGT C - 3' (forward) and 5'- GAA GAT GGT GAT GGG ATT TC - 3' (reverse). GM-CSF primers were designed and supplied by Qiagen Hilden.

The amplification conditions were: initial 15 minutes at 95°C (to activate HotStarTaq DNA Polymerase), then 45 cycles of PCR with denaturation at 94° C for 15sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. The conditions of melting Curve achieved were: initial 15sec. at 95°C, then from 55°C for 15 sec to 95°C for 15 sec., which was recommended routinely to perform in order to verify speciaficity and identity of the PCR products. Melting curve analysis is an analysis step built into the software of the LightCycler. PCR was performed on an ABI Prism 7700 sequence detector equipped with a 96-well thermal cycler. Data were collected and analyzed with Sequence Detector v1.6 software (PE Biosystems). GM-CSF expression was normalized by comparison with the expression of the housekeeping gene GAPDH. Standard curves for both genes were constructed for each run using serial dilutions of GM-CSF and GAPDH cDNA with known copy numbers. The gene copy number in each sample was calculated using the standard curve equation ($y = - mx + b$) which describes the correlation of C_T values and copy number of the dilution curve. The mRNA expression levels of GM-CSF were calculated as follows:

$$\Delta C_T \text{ (sample)} = C_T \text{ target gene} - C_T \text{ reference gene}$$

$$\Delta C_T \text{ (calibrator)} = C_T \text{ target gene} - C_T \text{ reference gene}$$

$$\Delta\Delta C_T = \Delta C_T \text{ (sample)} - \Delta C_T \text{ (calibrator)}$$

If the PCR efficiencies of the target gene and endogenous reference gene are comparable, the normalized level of target gene expression is calculated by using the formula:

Normalized target gene expression level in sample= $2^{-\Delta\Delta C_T}$;

3.2.8 Apoptosis measurement

Apoptosis was measured using the Annexin V-FITC apoptosis detection kit according to manufacturer's recommendations. Briefly, 1-5×10^5 cells were collected and washed once with PBS, then stained with 5µl FITC conjugated annexin V antibody in 195µl 1× binding buffer. The reaction was carried out at room temperature for 10 minutes. Unbinding annexinV antibody was removed by washing with 1× binding buffer. The cells were resuspened in 1× binding buffer and applied to the FCAScan immediatedly after stained with 20ug/ml propidium iodide (PI). PI and annexin V double negative cells were recognized as surviving, Annexin V positive and PI negative cells as early apoptosis, PI positive part was counted as late apoptosis.

3.2.9 Proliferation

Cell proliferation was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromid (MTT) colorimetric reduction method as described by the manufacturer (Sigma Chemical). Absorbance at 570nm was measured in an OptiMax microplate reader (Molecular Devices, Ismaning, Germany). Alternatively, proliferation was also measured using an MTS (3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium)-based method by absorption of formazan at 492 nm (CellTiter 96; Promega, Madison, WI). Measures were taken as quadruplicates after 24-48 hours of incubation with reagents. Briefly, 100ul 7.5×10^5/ml treated cells were added into 96-well-plate in triplicate and incubated at 37°C, 5% CO_2 for 24- 48 hours. 20µl of Cell Titer 96 Aqueous One Solution Reagent were piped into each well of the 96-well plate containing the samples. Followed by another incubation of 1-4 hours at 37°C in a humidified, 5% CO_2 atmosphere, brown colour developed and absorbance at 492nm was measured.

3.2.10 Cell cycle analysis

1 x 10^6 cells were harvested and washed once with PBS. Resuspended in 500µl PBS, cells were fixed by dropping 3 ml 70% cold ethanol (stored at -20°C) while vortexing and stored at 4°C for overnight. Before measurement, samples were washed once with PBS and incubated in 1 ml PBS containing 50µg PI and 200µg RNase for 30 minutes at room temperature. Following incubation, cell cycle was analyzed by FACS machine.

3.2.11 Akt kinase assay

The Akt activity was measured using Akt Kinase Assay Kit in which GSK (glycogen synthase kinase-3)-fusion protein was used as a substrate of Akt kinase. The phosphoryaltion level of GSK3 represents the activity of Akt. Briefly, whole cell protein extracts from 3×10^6 LAMA cells were reacted with immobilized anti-Akt antibody by gently rotating for overnight at 4 °C. Beads were washed twice with 1× cell lysis buffer, twice 1× kinase buffer and resuspened in 1× kinase buffer supplemented with GSK-3 fusion protein and ATP. The reaction was carried at 30°C for half hour. Terminated reaction with 25µl 3× SDS buffer and heated it at 95°C for 5 minutes, 15µl supernant was separated by 7.5% SDS polyacrylamide gel. The phosphorylation of GSK-3 was detected with anti-phospho-GSK-3.

3.2.12 Akt-1-siRNA-transfection

1×10^6 LAMA cells were palleted and resuspended in 100µl solution V (a cell line specific Nucleofector kit V). 2.5 µg human Akt-1-specific siRNA and equal amount of negative control siRNA were respectively mixed with the cells and added into the cuvette gently to avoid any bubble. The transfection was performed with the Amaxa Nucleofector Device (program T-16) according to manufacturer's recommendations. Western blotting and apoptosis were assessed at 48hours after transfection.

3.2.13 Mutagenesis screen

4×10^5/200µl Ba/F3-Bcr/Abl (p185) wild-type cells and parental LAMA cells were cultured in 96-well plate in presence of imtinib or nilotinib at 2µM combining with or without 0.1nM and 0.5nM everolimus; rh-GM-CSF 0.1, 0.25, 0.5ng/ml; AG490 100µM. The concentrations of imatinib and nioltinib were increased to 3µM after 72 hours and 4µM after 96 hours. Following 10-14 days' culture, single colonies growing out on the bottom of the well were counted.

3.2.14 Generation of conditioned medium

Conditioned media were generated by respectively culturing 1×10^6 /ml LAMA-cells in serum free RPMI-1640 medium (Gibco-BRL) and 1×10^6 /ml PBMC in serum free IMDM-medium

(HyClone) containing 4mM L-Glutamine and 0.1µM HEPES for 24h in a humidified atmosphere at 37°C with 5% CO_2. Supernatant was cleared from contaminating cells by centrifuging twice at 1500rpm for 5 minutes. Supernatant was eventually aliquoted and stored at -80°C for later use.

3.2.15 CFC assays

First, 5×10^5 $CD34^+$-enriched primary CML progenitors of untreated chronic phase CML patients (#3 to #8, Supplementary Table 1) were treated as indicated in conditioned medium of LAMA cell clone 25R (or 25UR-CM as control) in presence of 10µM NI for 72h. In some experiments $CD34^+$-enriched primary CML progenitors were treated with 10µM NI for 72h in CM derived from primary PBMC of IM-resistant patients. Conditioned media were always supplemented with a five growth factor cocktail (5GF) containing GM-CSF (0.2ng/ml), SCF (0.2ng/ml), IL-6 (1ng/ml), G-CSF (1ng/ml), and MIP-1α (0.2ng/ml). Alternatively, $CD34^+$-enriched primary CML progenitors were cultured in IMDM-medium (4mM L-Glutamine, 0.1µM HEPES) containing the 5GF plus 20% fetal calf serum (FCS) (Gibco-BRL), and treated with 10µM NI plus/minus additional AG490 (100µM) for 72h. To test the effect of GM-CSF, G-CSF, or IL-3 (all from Peprotech) on CFC formation, these cytokines were each individually supplemented at 10ng/ml. After treatment, cells were transferred into semisolid Methocult™-Medium (Stemcell Technologies Inc., H4230) and incubated in triplicates for 14 days in 35×10mm plates (Greiner Bio-One, Frickenhausen, Germany) at 37°C in a humidified atmosphere containing 5% CO_2 saturation. Colonies were counted under microscope.

3.2.16 Cytokine antibody array

A cytokine antibody array was performed using RayBio® Human Cytokine Antibody Array C Series 1000 Kit (Ray Biotech, Norcross, GA) according to manufacturer's recommendations. Briefly, two membranes each consisting of 60 cytokine antibodies spotted in duplicates onto the membranes were blocked with 2ml 1X blocking buffer at room temperature for 30min. Membranes were then incubated with 2ml serum free conditioned media derived from LAMA subclones 25UR and 25R, respectively, at room temperature for 90 min. Membranes were washed three times for 5 minutes at room temperature with 2ml of 1X wash buffer I, and two times with 2ml of 1X wash buffer II according to the manufacturer's recommendations. Membranes were then exposed for 90 min at room temperature to 1ml of a 1:500 dilution of biotin-conjugated antibodies. Following a thorough wash as before, membranes were incubated with 2ml 1:1,000 diluted HRP-conjugated

streptavidin at room temperature for 120 min. Membranes were washed again and exposed for 2 minutes to the peroxidase substrate, which was constituted by mixing 1X detection buffer C and 1 x Buffer D in a 1:1 ratio. Membranes were exposed to BioMax-MR films (Eastman Kodak, Rochester, NY) for appropriate times.

3.2.17 Intracellular staining

For intracellular staining, 1 to 5×10^5 stimulated parental LAMA cells or primary $CD34^+$ enriched CML samples were fixed at 37°C for 10min using Fix Buffer I (BD Biosciences, Heidelberg, Germany) and then immediately stored at -80°C for future analysis or permeabilized cells on ice for 30min in 1ml BD™ Phosflow Perm Buffer III (BD Biosciences), washed twice with BD Pharmingen™ Stain Buffer, resuspended in 100µl BD Pharmingen™ Stain Buffer and added as indicated fluorochrome-conjugated antibodies: 5µl anti-CD34 PE-Cy7 (8G12), 5µl anti-p-STAT-5-PE (Y694), 5µl biotinylated mouse anti-human GM-CSFR (CD116, 4H1), 2.5µl anti-p-CrkL (Y207), and 2.5µl anti-p-Jak2. Labelling occurred for 30 minutes at room temperature in the dark. After first staining, cells were washed twice with BD Pharmingen™ Stain Buffer and secondary staining was performed with 3µl fluoreszeinisothiocyanat (FITC)-labelled goat anti-rabbit IgG and 3µl Streptavidin-APC. Cells were again washed and resuspended in 500 µl of BD Pharmingen Stain Buffer for FACS analysis on a LSR II FACS-analyser. Data were analyzed using FlowJo software.

3.2.18 Human GM-CSF ELISA assay

GM-CSF concentration in total protein cell lysates generated from peripheral blood cells of IM-sensitive and -resistant patients (Supplementary Table 3) was quantitated using the Human GM-CSF ELISA-kit from Diaclone (Besançon, France); detection range: 8pg/ml-500pg/ml, sensitivity < 4.4pg/ml. Briefly, microtiter wells were washed twice with washing buffer. 30µg cell lysate was added per well - for each sample in duplicate. Horse radish peroxidase (HRP) conjugate (HRP-conjugated anti-GM-CSF monoclonal antibody) was added followed by an incubation on a plate shaker for 3 hours at room temperature. Subsequently, wells were washed; 100µl TMB substrate solution was added and the plate incubated for 20 minutes at room temperature. Finally, 100µl stop solution was added and the absorbance was measured on an ELISA Reader (Molecular Devices, Ismaning, Germany) at 450nm. The absorbance values of the samples were converted into concentration values based on the calibration curve.

3.2.19 Statistical analysis

Analysis of significance of differences in treatment groups were performed using GraphPad Prism 4.02 Software. One-way ANOVA analysis was used with Dunnetts or Bonferroni adjustments for multiple comparisons or Mann Whitney U testing for comparison of less than two treatment groups. A p-value < 0.05 was considered as significant.

4. RESULTS

4.1Compensatory PI3-kinase/Akt/mTor activation regulates IM resistance development

4.1.1 IM-induced Akt/mTor-activation mediates survival before emergence of strong IM-resistance in vitro.

To generate IM-resistant cell clones, IM-naïve individual LAMA-clones were exposed to increasing concentrations of IM (+IM) or alternatively mock treated as control (-IM) (**Figure 7**). Cell lysates, cell aliquots and total RNA of each clone were monthly preserved during the course of IM-resistance induction for later analysis. Two clones, resistant to > 1μM IM, were analyzed for the role of Akt activation as a putative early mechanism of IM resistance: (i) clone 14+IM, which developed a clinically relevant BCR/ABL-kinase point mutation Gln252His (**Figure 8A.a**) and (ii) clone 10+IM with a BCR/ABL-dependent, unknown mechanism of IM-resistance since no gene amplification and BCR/ABL mutation could be detected.

In clone 14+IM, mutated Gln252His-cDNA traces were first detected at day 80 (d80) (9 % mutated signal) and increased to 72% at d110, suggestive of clonal expansion (Figure 4.1.1A, b). Analysis of cryo-preserved aliquots of clone 14+IM derived from days 0, 80, 110 and 140, respectively, revealed that strong IM-resistance occurred only after day 110+IM, that is, after expansion of Gln252His-mutated cells to >70% (**Figure 8B**). BCR/ABL expression remained unchanged in clone 14+IM throughout the experiment (not shown). Hence, IM-resistance/survival of clone 14 before d110 was due to mechanisms other than the Gln252His-point mutation or BCR/ABL overexpression. When compared to d0, survival of cells from d50+IM (**Figure 8C, a, b**) could be attributed to a great extent to an activation of the anti-apoptotic PI3K/Akt-pathway, because d50+IM cells were only killed in the presence of IM after the PI3K-inhibitor wortmannin (WM) was added (**Figure 8C, b**). In line with this biological observation, only the co-administration of IM and WM, but neither of the drugs alone, blocked Akt-phosphorylation (p-Akt) and Akt kinase activity, as assessed by means of phosphorylation of the Akt-specific substrate GSK3α/β (**Figure 8C, b**). Notably, clone 14 lost its strong dependence on PI3K for survival in presence of IM at d180 (with > 80% Q252H-positive cells in culture), suggesting stage-specific roles for this pathway during manifestation of IM-resistance.

Clone 10 +IM developed BCR/ABL-dependent IM resistance around d220 (**Figure 9A** and **B**). BCR/ABL overexpression (**Figure 9B**) as well as BCR/ABL-kinase mutation (not shown) was

excluded as resistance mechanism. Akt activation again occurred before emergence of strong IM resistance at d220 (**Figure 9C**), and contributed to IM resistance: SH-6, a novel selective Akt inhibitor (Kozikowski AP. et al., 2003), killed clone 10 only at d180 but not at d220 (**Figure 9D**). This illustrated that clone 10 particularly depended on Akt-signaling for survival at d180, but not at d220 (**Figure 9D**). These findings were validated using Akt1-specific siRNA. Akt1-siRNA equally down-regulated Akt1 at d180 and d220 (**Figure 9E**, upper panel), but apoptosis was augmented only at d180 (**Figure 9E**, lower panel). Stronger IM resistance at d220 was associated with an increased phosphorylation of p70S6K (pT389p70S6K) compared with d180 (**Figure 9E**), indicating that activated p70S6K contributed to stronger IM resistance at d220.

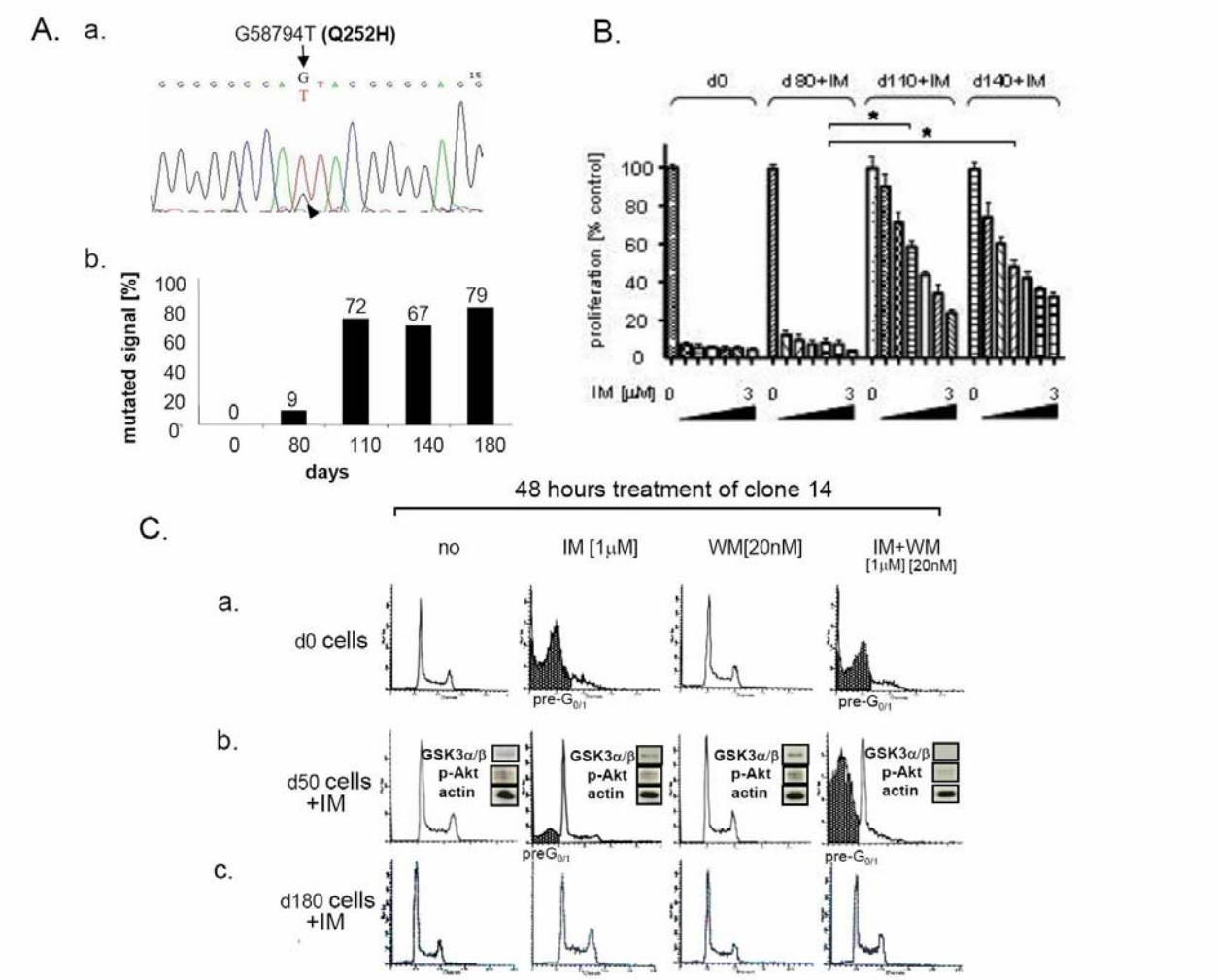


Figure 8 PI3K/Akt-mediated survival prior to the development of IM-resistance through a de novo occurring BCR/ABL-kinase mutation

A. The G58794T mutation in the P-loop of BCR/ABL leads to the amino acid exchange Q252H. a) Primary sequence data of clone14 on day 210+IM are shown. b) Quantitation of mutated DNA signal traces before day 210+IM. B. Assessment of IM-resistance by MTT- colorimetric assay. Statistically significant differences ($P<0.05$) in proliferation-inhibition at different days were assessed using the Mann-Whitney test and are indicated by asterisks (*). Cell aliquots derived from indicated time points of IM resistance induction in clone 14. Cell samples were treated for 48h with increasing concentrations between 0.5 and 3µM of IM. C. Cell cycle analysis of cell cultures of clone 14 derived from d0 (a), d50 (b) and d180 (c). Cells were treated for 48h-treatment with indicated compounds; p-Akt protein levels (detected by p-Akt-specific antibodies) and the Akt kinase activity on d50+IM as measured by means of phosphorylation of the Akt-specific substrate GSK3α/β are shown in the middle panel. The pre-G0/1 peaks represent apoptotic cells (black).

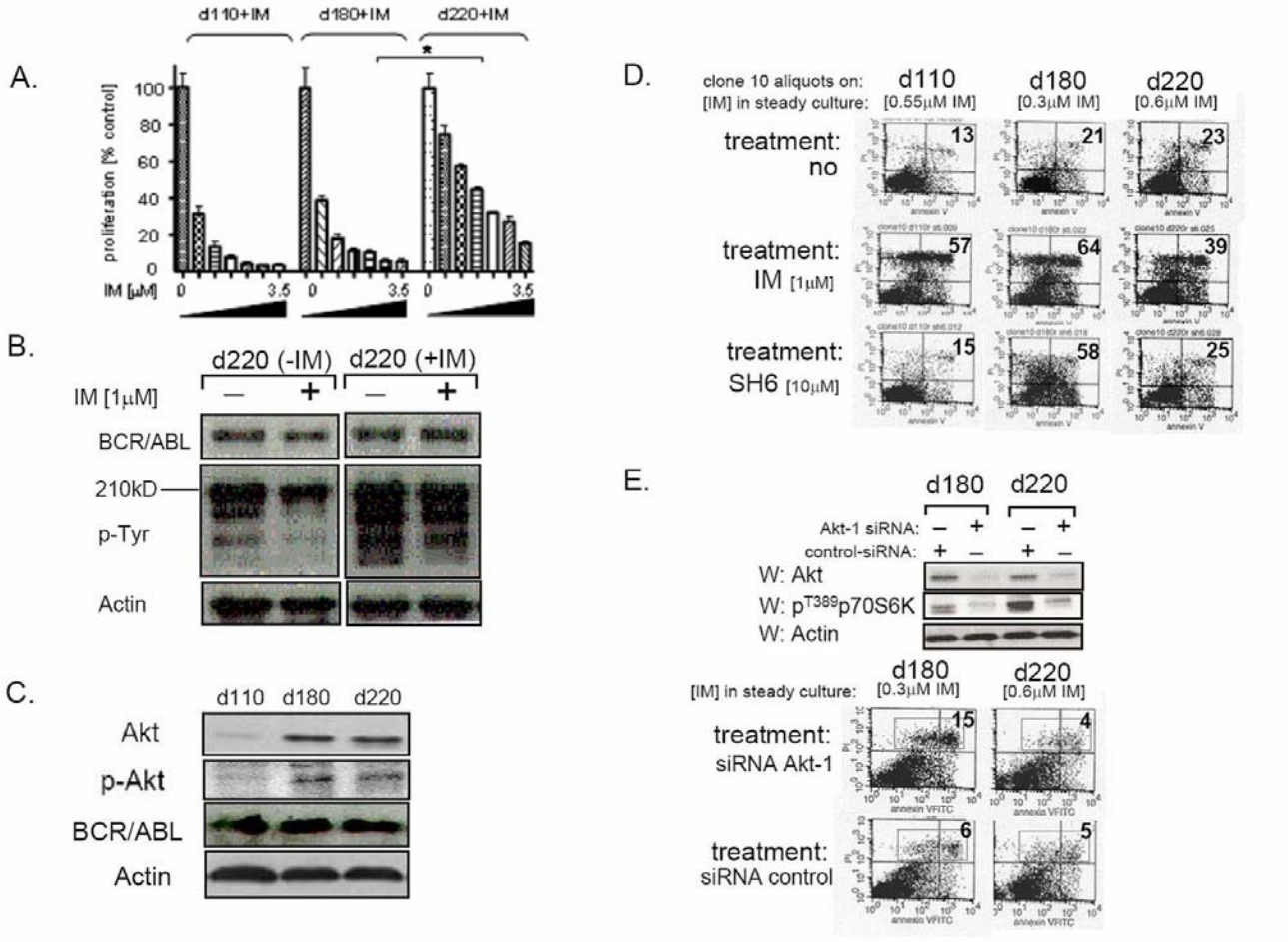


Figure 9 Akt activation mediates incipient IM-resistance in clone 10

Biochemical and biological changes during IM-resistance development of clone 10. A. Assessment of IM-resistance using an MTT-colorimetric assay. Two independent experiments were performed. Values are expressed as mean of five replicates. Bars: ±s.d. B. Anti-BCR/ABL- and anti-phosphotyrosine-western blotting. Whole protein extracts were generated from clone 10 at d220 +IM and the parallel IM-naïve control cells (d220-IM) after treatment with 1µM IM or mock treatment for 24h. C. Anti-Akt- and anti-p-Akt western blotting of whole cell lysates derived from preserved lysates of clone 10+IM from d110, d180, and d220. D. AnnexinV-FITC/PI-staining-based apoptosis measurement of clone 10-aliquots from d110, d180, and d220 of IM-resistance induction after a 48h-treatment with IM, SH-6 or mock-treatment. Percentages of apoptotic cells are depicted in the right upper quadrants. A representative experiment of two independent experiments is shown. E. Upper panel: Anti-Akt- and anti-pT389p70S6K-western blotting of Akt1- and control-siRNA treated clone 10-aliquots from d180 and d220. Lower panel: increase in apoptosis by Akt1-specific siRNA compared to control-siRNA as assessed by AnnexinV-FITC/PI-staining. Four independent experiments were performed. Bars: ±s.d. ´*´ indicates statistically significant differences.

4.1.2 Compensatory activation of Akt and p70S6K contributes to survival and IM resistance development

Compensatory activation of the PI3-kinase/Akt/mTor-signaling may contribute to incipient IM-resistance. To recapitulate and further validate this finding, clone 10 and 14 were again exposed to IM or, alternatively, to IM and Rap. Neither clone 10, nor clone 14 developed a kinase mutation in this experiment (not shown). In analogy to the results above (**Figure 8, 9C, E**), IM treatment prompted an activation of Akt (clones 10 and 14), or the downstream target of Akt, p^{T389}p70S6K (clone 10) (**Figure 10A**). Notably, mTor-inhibition using Rap prevented Akt-activation by IM (**Figure 10A**) and retarded IM-resistance development (**Figure 4.1.3B**). Similar results were obtained in two primary cell samples (**Figure 10C** and **D**). IM at 0.5 μM, administered for up to 9 days, activated Akt and p70S6K in IM-naïve CML blasts, but did not increase apoptosis (**Figure 10C**). In turn, Rap antagonized IM-induced Akt activation, resulting in apoptosis (**Figure 10C**). Cells derived from another CML patient in chronic phase, who lost hematological response due to incompliant IM intake (irregular and reduced IM-doses), displayed high p^{T389}p70S6K levels and a constitutive phosphorylation of Akt (**Figure 10D**). Treatment of these cells with 1μM IM for 48h only partially inhibited p^{T389}p70S6K, but neither affected p-Akt levels, nor apoptosis (**Figure 10D**). In contrast, 10nM Rap for 48h completely inhibited p-AKT and p^{T389}p70S6K and caused significant apoptosis (**Figure 10D**). Altogether, these data are in line with the concept that IM-naïve BCR/ABL-positive cells employ a compensatory Akt/mTor-signal activation to escape BCR/ABL inhibition through IM.

4.1.3 The mTor-inhibitior RAD001 inhibits IM resistance development in a novel cell-based resistance induction assay

If Akt/mTor signaling is predominantly required for incipient IM resistance, we reasoned that mTor inhibition would block IM resistance development also in a novel recently reported cell-based strategy for induction of inhibitor resistance (Von Bubnoff N. et al., 2004). In this assay, an alternative mTor inhibitor, RAD001 (Huang S. et al., 2003) was used. RAD001 (unlike rapamycin) is being developed in oncology and is clinically well tolerated (Eisen HJ. et al., 2003). RAD001 dose dependently reduced the frequency of IM-resistant colonies by 59 % and 94% of the IM control, respectively (**Figure 11A**). This strikingly potent efficacy of RAD001 on colony formation may be explained only to some extend by its growth-inhibitory effects, because low

doses of RAD001 only marginally inhibited proliferation of Ba/F3-BCR/ABL cells (**Figure 11B**) and did not affect apoptosis in short-term cultivation experiments (**Figure 11C**).

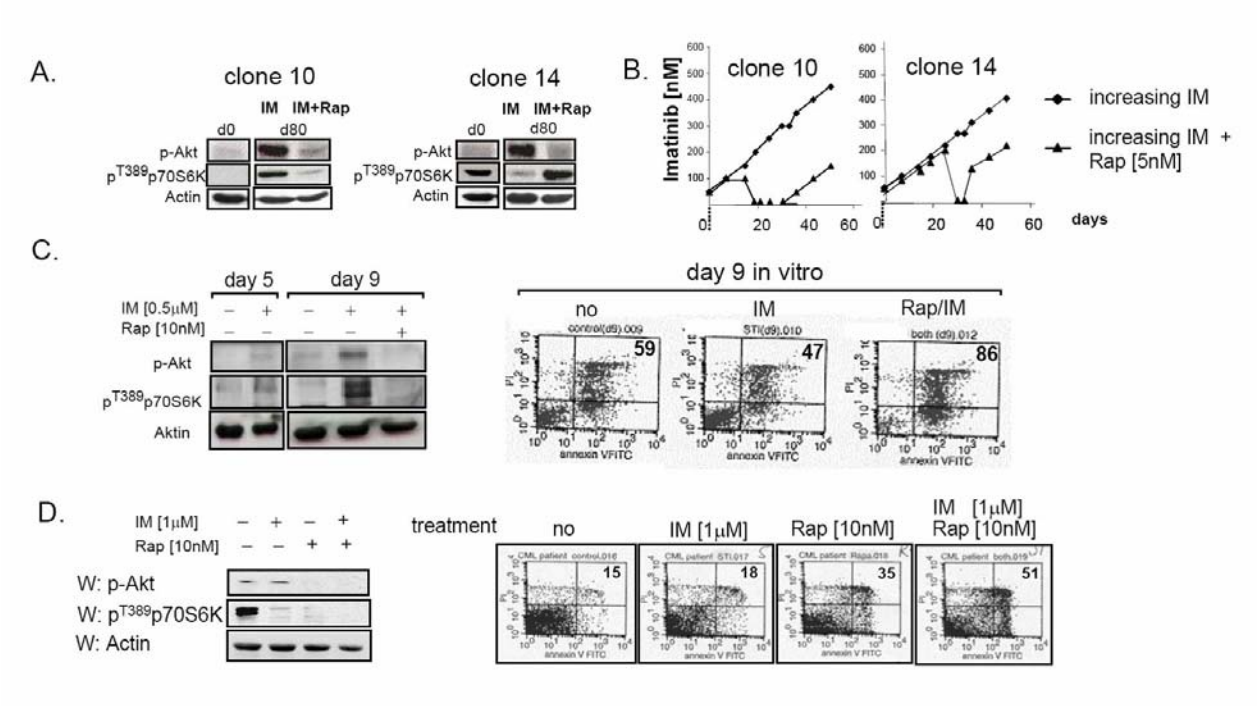


Figure 10 Rap inhibits IM-induced activation of Akt/mTor-signaling and antagonizes IM-resistance development

A. Western blotting of protein lysates harvested before and after 80 days of culturing IM-naïve clones 10 and 14 with increasing concentrations of imatinib (IM), or a combination of increasing concentrations of IM and steady 5nM Rap (IM+Rap). B. Corresponding resistance induction dynamics of clones 10 and 14 as indicated. Dots indicate the tolerated IM-dose (with ≥ 30% viability) at indicated times. C. In vitro treatment with IM or IM plus Rap (5nM) of primary blasts of an IM-naïve patient, who relapsed with a lymphatic blast crisis. Left: Western blotting of whole protein extracts after five and nine days, respectively, of in vitro culture in presence of IM and/or Rap as indicated. Right: Corresponding analysis of apoptosis 9 days after treatment using Annexin-FITC/PI staining. The total percentage of early and late apoptotic and necrotic cells is shown in the upper right quadrant. D. Western blotting of Akt and p70S6K of primary peripheral blood leukocytes of a chronic phase CML patient under IM-therapy after in vitro treatment with IM or Rap as indicated. Left panel: p-Akt and pT389p70S6K-western blotting. Right panel: corresponding AnnexinV-FITC/PI apoptosis measurement as in left panel.

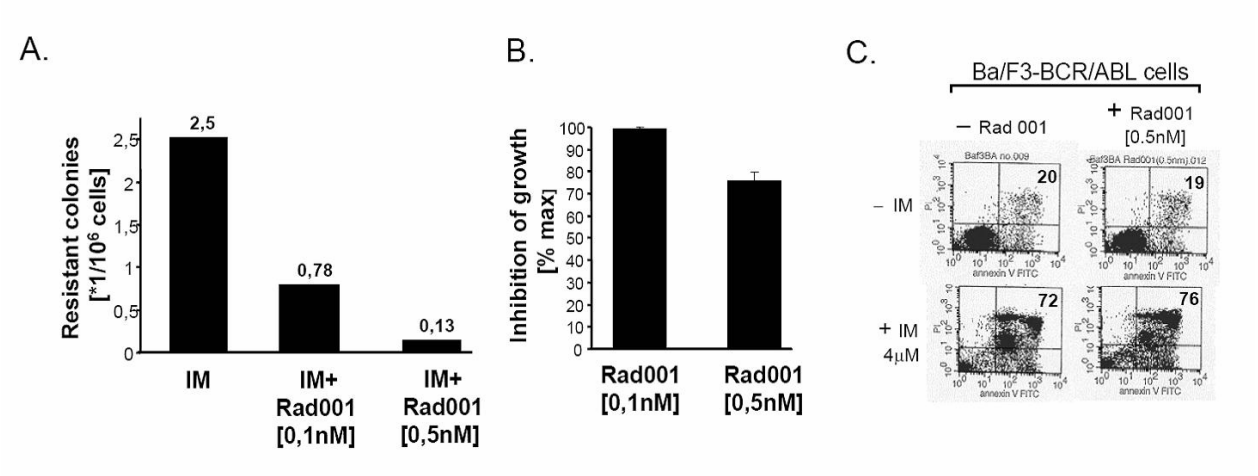


Figure 11 mTor-inhibition blocks IM-resistance development in a cell-based IM-resistance induction assay

A. Frequency of resistant colonies emerging in the presence of IM without and in the presence of everolimus. The number of colonies growing in the presence of IM alone or IM and the mTor inhibitor everolimus at the indicated concentrations is expressed as frequency of resistant colonies in relation to the number of Ba/F3-BCR/ABL wild-type cells.B. Inhibition of cell growth by everolimus using an MTS tetrazolium-based assay. Two independent experiments were performed. Values are expressed as mean of triplicates. Bars: ± s.d. representative results of one experiment are shown. C. Apoptosis measurement using AnnexinV-FITC/PI-staining after 48h treatment with indicated compounds. Bars: ± s.d. three independent experiments were performed.

4.1.4 Heterogeneous activation of Akt and p70S6K in IM-resistant patients with BCR/ABL kinase mutation

We reasoned that PI3K-signaling may be particularly important for early IM resistance. Blockage of mTor has been shown to overcome manifest IM resistance in vitro and in animal studies, and blocking of an activated Akt-/mTor-signaling has been suggested as a means to overcome clinical IM resistance. Here, we analyzed the activation status of Akt and mTor in CML patients (n=17) with clinically overt IM resistance through kinase mutations (n=15). Each of the 17 tested patient samples was derived during clinical IM resistance, with a 100% Ph+-status as mirrored by the high BCR/ABL-ABL mRNA ratios ranging between 40-100% (**Figure 12A**). All patients were on IM (for indicated times) at the time of sample acquisition (**Figure 12A**). In spite of the presence of a mutated BCR/ABL kinase, only a minority of the patients displayed an activation of the downstream targets of BCR/ABL, Akt (n=7), and p70S6K (n=8), which is downstream of Akt (**Figure 12A**). Even patients harboring the same mutation, such as the Tyr253His-mutation in

patients #1, #3, #4, #5, and #6, showed inconsistent Akt, p-Akt and p ^{T389}p70S6K -expression levels (**Figure 12A**). There was also no clear correlation between Akt expression, Akt phosphorylation, and p70S6K-activation, indicating no linear activation mode of this pathway during manifest IM resistance (**Figure 12A**). Hence, Akt/mTor activation occurs independently from BCR/ABL and may therefore variably contribute to overt IM resistance in vivo. We therefore tested two successive IM-resistant patients with acute lymphatic leukemia (ALL) and found that only the patient with strong p ^{T389}p70S6K-expression and high levels of p-Akt (**Figure 12B, a**) responded to Rap in vitro, whereas the second patient with low levels of p ^{T389}p70S6K and p-Akt did not respond to Rap treatment in vitro (**Figure 12B**). Susceptibility to Rap in patient #18 was associated with a decrease of p-Akt and p ^{T389}p70S6K (**Figure 12B**). This suggests that the variable degree of Akt/mTor-activation in IM-resistant patients with CML or ALL Akt activation may predict responsiveness to mTor-inhibitor treatment.

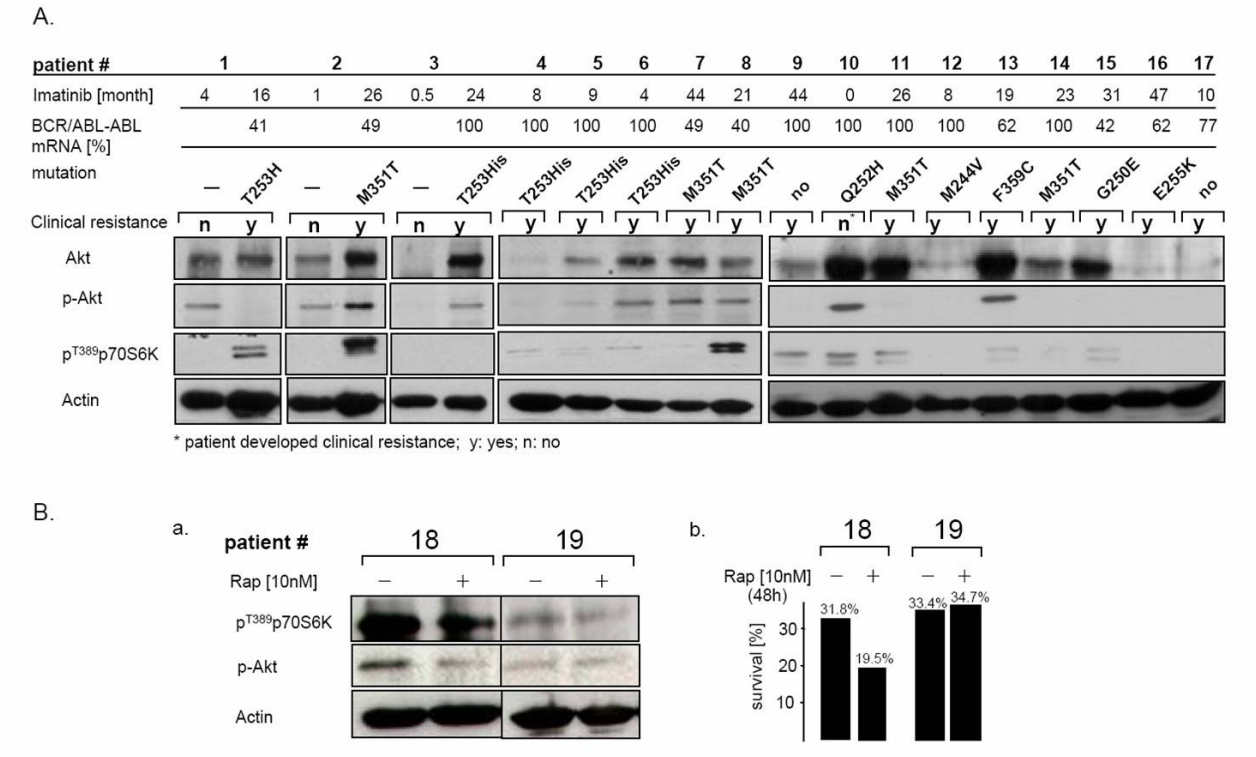


Figure 12 Heterogeneous activation of Akt-signaling in patients with clinically overt IM resistance though BCR/ABL-kinase mutations.

A. Western blotting of peripheral blood lysates of CML-patients with clinical IM resistance through BCR/ABL kinase mutations. Duration of IM treatment prior to emergence of clinical IM resistance is indicated. The quantitation of the BCR/ABL-mRNA levels at the time of clinicalIM resistance is shown. Membranes were first blotted with anti-p-Akt antibody, stripped and re-probed with anti-Akt, anti-p^{T389}p70S6K, and finally with anti-Actin antibody to ensure equal loading. B. Akt-activation status and response to mTor-inhibitor treatment in two IM-resistant All patients (18 and 19). (a) Western blotting. Peripheral blood cell lysates were generated after a 48h treatment with Rap (10nM) or mock-medium and blotted with anti-Akt antibody. Membranes were reprobed with anti-p^{T389}p70S6K and anti-actin antibody to ensure equal loading. (b) Parallel Annexin V-FITC/PI-staining-based apoptosis measurement. The percentage of surving cells of patients 18 and 19 after 48h of Rap-treatment (Annexin V-FITC and PI-negtive) is depicted and compared to mock-treatment, respectively.

4.2 Adaptive secretion of the Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) mediates IM- and NI-resistance in BCR/ABL-positive progenitors via JAK-2/STAT-5 pathway activation

4.2.1 Conditioned medium of IM-resistant LAMA clones mediates IM and NI resistance

IM at 1µM induces almost complete apoptosis of parental LAMA cells. To test the effect of culture supernatant (conditioned media, CM) of IM-resistant LAMA-84 cell clones (Burchert A. et al., 2005) (R-CM) on IM- or NI-induced apoptosis, R-CM was applied to IM-naïve LAMA-84 cells in presence of 1µM IM or NI. Intriguingly, R-CM of the IM-resistant LAMA-cell clones, 14R (harboring a Q252H point mutation), 10R and 25R potently protected from IM-, but also NI-induced apoptosis ($p< 0.001$) (**Figure 13A**). In contrast, plain FCS or sensitive CM (UR-CM) derived from IM-naïve homologue clones (10UR, 14UR and 25UR), or R-CM of another IM-resistant LAMA-clone (2R) did not protect from IM induced apoptosis.

To ask, whether R-CM induced IM- and NI-resistance exclusively in LAMA cells, we next also tested R-CM on K562 cells and primary $CD34^+$CML progenitors. It has previously been established, that IM and DA selectively inhibit proliferation of CML–CFC in vitro (Copland M. et al., 2006; Holtz MS. et al., 2002; Deininger MW. et al., 1997). Although inefficient in K562 cells (not shown), 25R-CM clearly overcame NI-mediated growth inhibition of primary $CD34^+$-enriched progenitor samples from first diagnosis chronic phase CML patients (n=3). This was illustrated by a 6.9-fold (±3.5 s.d) increase in the number of CML-colony forming cells (CFC, colonies) surviving NI-exposure through presence of 25R-CM instead of 25UR-CM (**Figure 13B, Table 1**). Notably, also CM derived from an IM-resistant patient (pat. #1, **Table 1**) was (87-fold) more potent than 25UR-control CM in overcoming CFC inhibition by NI (**Figure 13B**). Together, these experiments suggested that factors were secreted by IM-resistant LAMA cells and primary cells which promoted IM and NI resistance.

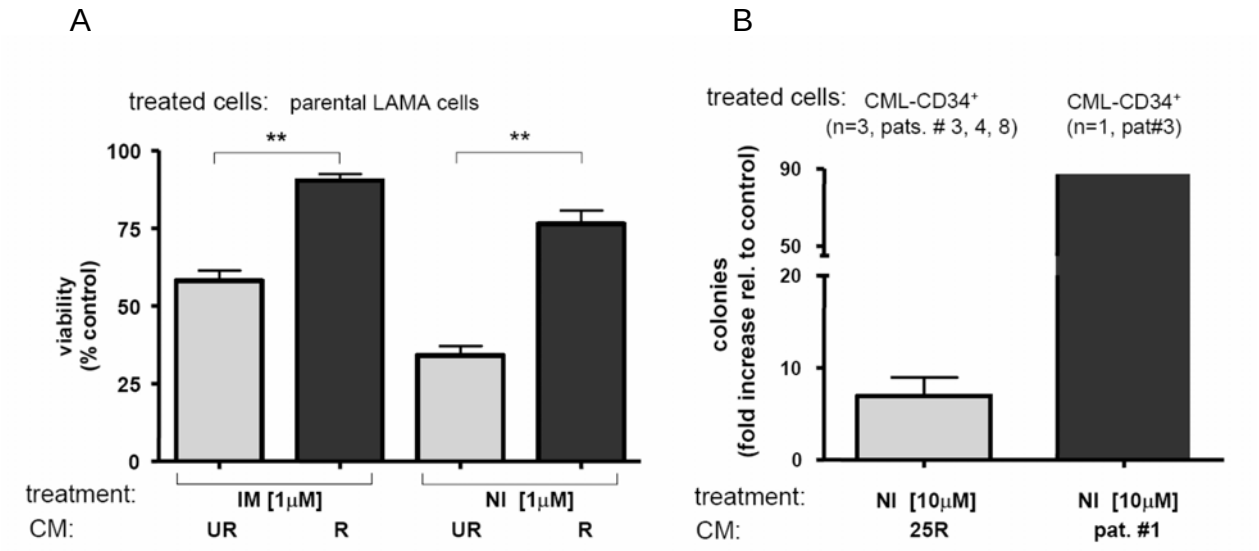


Figure 13 Conditioned medium of IM-resistant LAMA cells confers IM and NI resistance to IM-naïve LAMA-cells

A. CM of IM-resistant (R) clones protects IM-naïve LAMA cells from IM- and NI-induced apoptosis. Percentage of viable cells after treatment is shown relative to no treatment controls. Bars represent mean ± s.d. of three independent experiments using CM of the three R- and three UR-clones (10, 14 and 25), respectively; ** $p < .001$ (one-way ANOVA, Bonferroni adjustment for multiple comparisons). B. 25R-CM and pat.#1 derived CM protects IM-naïve, $CD34^+$-enriched progenitors of first diagnosis CML patients (n=3, pat. #3, #4, #8). Primary progenitor cells were exposed to 10μM NI for 72h in presence of 25UR-CM (as control), 25RCM, or IM-resistant pat. #1-derived CM (black bar) and then placed into semisolid Methocult™-medium. Emerging colonies were counted. Bars represent fold increased colony formation ± s.d. by 25R-CM (grey bar), or pat. #1-derived CM (black bar) relative to 25UR control treatment.

4.2.2 R-CM mediates BCR/ABL-independent NI-resistance

Next we asked if 14R and 25R-CM-induced IM/NI resistance was BCR/ABL-dependent. BCR/ABL-kinase activation status was assessed using western blotting for detection of phosphorylated BCR/ABL (p-BCR/ABL) and Crk-like protein (p-CrkL). Activation of another key BCR/ABL-substrate, signal transducer and activator of transcription (STAT)-5, was also evaluated (**Figure 14**). Neither R- nor UR-CM interfered with the inhibition of BCR/ABL by IM or NI (**Figure 14**). In fact, NI completely dephosphorylated BCR/ABL (and CrkL) indicating that 14R/25R-CM-mediated resistance was BCR/ABL-independent. In contrast, STAT-5 phosphorylation was exclusively blocked in presence of 14UR/25UR-CM (**Figure 14**), but not 14R/25R-CM. Thus, we concluded that 14R/25R-CM-induced IM and NI resistance must have

been associated with a BCR/ABL-independent activation of STAT-5.

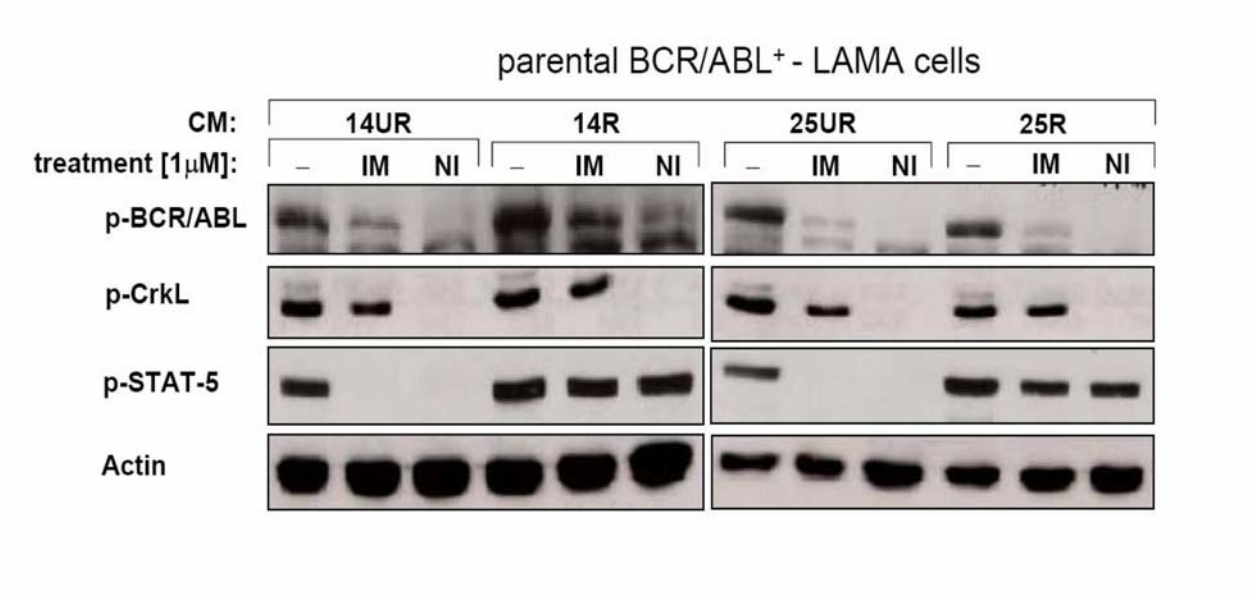


Figure 14 R-CM mediates BCR/ABL independent activation of STAT-5

Western blotting. 14R-CM and 25R-CM causes a BCR/ABL-independent activation of STAT-5 by in presence of IM or NI. Activated (phosphorylated) BCR/ABL kinase (p-BCR/ABL) was demonstrated by staining for p-BCR/ABL and the phosphorylated direct substrate of BCR/ABL, p-CrkL. Activation of the BCR/ABL-substrate, STAT-5, was shown using phospho-STAT-5 (p-STAT-5) specific antibodies. Actin served as loading control.

4.2.3 GM-CSF is causal for NI resistance induction by 25R-CM

STAT-5 is a common antiapoptotic and transforming target of BCR/ABL and janus kinases (JAKs) (Shuai K. et al., 1996; Horita M. et al., 2000; Yamaoka K. et al., 2004). Cytokines, upon binding to their receptors, activate members of the Jak family (e.g. Jak1, Jak2, Jak3 and tyrosine kinase 2, Tyk2) and subsequently STAT-5 (Yamaoka K. et al., 2004). We hypothesized that the resistance mediating factor in R-CM could be a cytokine. 120 cytokines were screened by cytokine array for differential expression between 25UR and 25R conditioned medium. The concentrations of granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-6 (IL-6), monocyte chemoattractant protein 1 (MCP-1), interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) were substantially increased in 25R-CM as compared to 25UR-CM (**Figure 15A**). However, only GM-CSF, when supplemented into 25UR-CM, conferred resistance of parental LAMA cells to nilotinib (**Figure 15B**), indicating that GM-CSF was sufficient to mediate NI resistance. In fact, adding anti-rhGM-CSF antibodies, which neutralize GM-CSF in 25R-CM caused a dose-dependent reduction of the capability of 25R-CM to confer NI resistance to LAMA

cells (**Figure 15C**). Moreover, addition of GM-CSF into 25UR-CM dose dependently restored the biochemical phenotype of 25R-CM, namely, a BCR/ABL-independent STAT-5-phosphorylation of parental LAMA-cells in presence of NI (**Figure 15D**). Together, GM-CSF was identified as being involved in conferring BCR/ABL-independent IM and NI resistance by 25R-CM.

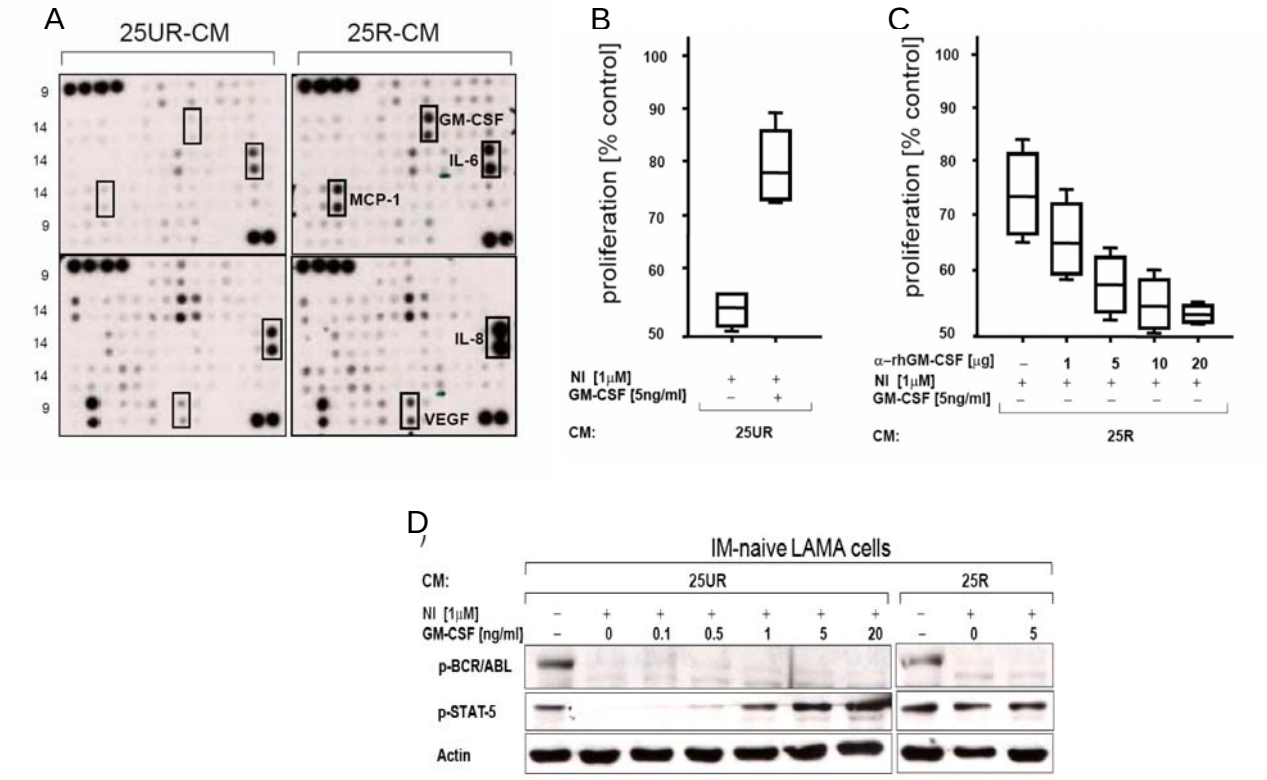


Figure 15 GM-CSF mediates BCR/ABL-independent IM and NI resistance

A. Cytokine array. 120 different cytokines were screened for differential expression in the CM of 25UR (left two membranes) versus 25R (right two membranes). 60 different cytokines are spotted in duplicate on each membrane. Positive controls (4 dots) are shown in the left upper parts. Darker spots indicate higher expression. Cytokines with increased expression in 25R-CM (bold lined squares) compared to 25UR-CM (thin lined squares) are indicated. B. The viability of parental LAMA cells after 24h of treatment with NI in presence of 25UR-CM and GM-CSF was assessed relative to the control treatment (no NI or GM-CSF) using MTS-colorimetric assay. Boxes display data points located in the middle two quartiles of all data points. Lines in boxes indicate medians; whiskers extend to the two extreme values of all data points. Data points of three independent experiments are depicted. C. Reversal of 25R-CM-mediated NI resistance of parental LAMA cells by addition of increasing concentrations of neutralizing anti-human-GM-CSF antibodies as indicated. Data points of three independent experiments are depicted. D. IM-naïve parental LAMA cells were treated with NI and increasing concentrations of GM-CSF as indicated in presence of 25R-CM or 25UR-CM. After 24h of treatment cells were harvested and cell lysates separated using PAGE and blotted with the indicated antibodies. Actin served as loading control.

4.2.4 BCR/ABL-independent activation of STAT-5 by GM-CSF in LAMA cells – role of CD116 expression

GM-CSF is a hematopoietic cytokine, regulating proliferation and survival of normal GM-progenitors (GMP) (Donahue RE. et al., 1986; 1988), but its aberrant secretion may also be involved in the pathogenesis of CML (Hariharan IK. et al., 1988; Sirard C. et al., 1994). The data above indicated that GM-CSF induced STAT-5 activation substitutes for the loss of BCR/ABL-mediated STAT-5 activation by NI. We sought to verify this on the single cell level by multi-color fluorescence activated cell sorting (FACS) using a combined intracellular and extracellular staining. Intracellular p-CrkL levels (ic-p-CrkL) as marker of BCR/ABL-activation and the ic-p-STAT-5 levels were concomitantly measured. It was confirmed that NI effectively blocked BCR/ABL in the presence or absence of GM-CSF (**Figure 16A**, upper histograms), and that STAT-5 inhibition by NI was overcome by GM-CSF (**Figure 16A**, lower histograms). We also noted that GM-CSF-receptor alpha chain (CD116) expression was critical for the regulation of STAT-5 by GM-CSF. An arbitrary discrimination between $CD116^{low}$ (yellow) and $CD116^{high}$ (blue) expressing LAMA cells revealed higher basal STAT-5 activation in the $CD116^{high}$ population (**Figure 16B**, i, right histogram), and a further increase of ic-p-STAT-5 by GM-CSF exclusively in $CD116^{high}$ cells (**Figure 16B**, ii, right histogram). On the contrary, CD116 expression did not influence NI-mediated p-STAT-5 inhibition in the absence of GM-CSF (**Figure 16B**, ii, left histogram). The scatter characteristics would support the conclusion that GM-CSF maintained viability (oval gate) in presence of NI preferably in the $CD116^{high}$ (blue), but not the $CD116^{low}$ population (yellow) (**Figure 16B**, iii) demonstrating that GM-CSF via binding to its receptor CD116 protected from NI induced apoptosis. In an attempt to address, whether GM-CSF supports long term survival of BCR/ABL positive cells in presence of NI or IM, we adapted a previously reported cell based in vitro resistance induction assay (Von Bubnoff N. et al., 2004). In this assay higher levels of IM (4 μM) were used to provoke resistance development (Von Bubnoff N., 2004; Burchert A. et al., 2005). Therefore, GM-CSF was added at variable concentrations to 96 well plates each containing $4x10^5$ cells in presence of NI or IM at 4μM. Readout after 14 – 30 days was the number of colonies that emerged (**Figure 16C**). This showed that GM-CSF dose dependently maintained survival, but also growth of LAMA cells in presence of high doses of IM or NI (**Figure 16C**).

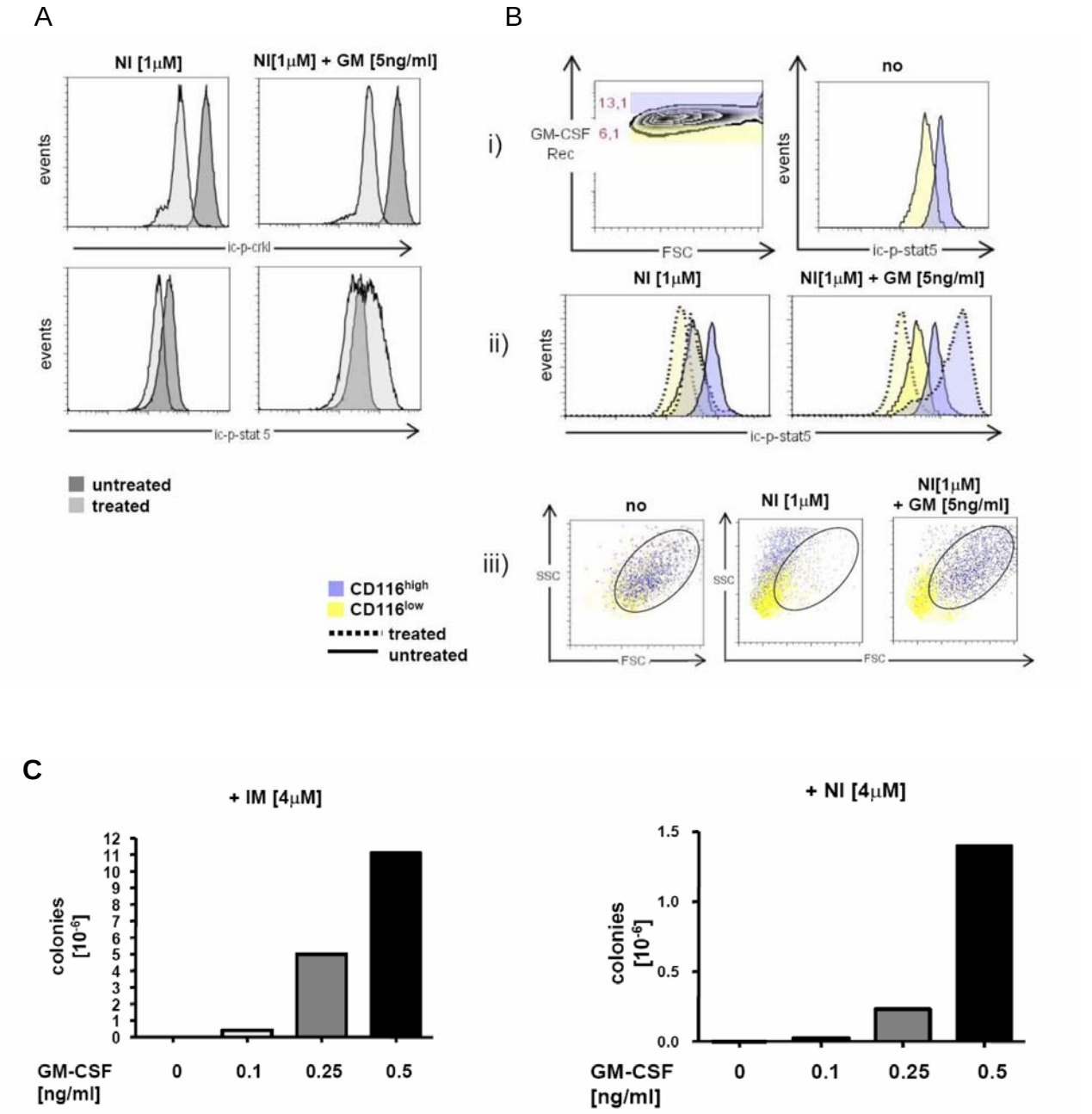


Figure 16 GM-CSF-receptor signaling mediates BCR/ABL-independent NI resistance through activation of STAT-5

A. Intracellular staining of p-STAT-5 (ic-p-STAT-5) and ic-p-CrkL in LAMA-cells before (dark) and after treatment (light) with NI or GM-CSF as indicated. B. Analysis of ic-p-STAT-5 according to GM-CSF-receptor alpha (CD116) expression levels; i) blue - denotes high CD116 expressers and yellow - low CD116 expressers; ii) ic-p-STAT-5 regulation according to high and low CD116 expression levels (blue and yellow) before (full lines) and after (dotted line) treatment with NI or NI plus GM-CSF. (iii) Viability of $CD116^{high}$ and $CD116^{low}$ populations of LAMA cells after exposure for 24h to NI plus/minus GM-CSF. The circle indicates viable cells according to the typical scatter

characteristics of viable LAMA cells. C. GM-CSF maintains growth and survival of LAMA-cells in presence of high doses of IM and NI. LAMA cells ($4x10^5$ cells / well) were cultured in 96 well plates and exposed to a final concentration of 4µM IM or NI in presence or absence of GMCSF as indicated. Colonies formed after 14 to 30 days and are depicted as resistant colonies per one million input cells at the beginning of the culture. One of three experiments is shown, respectively, showing very similar results.

4.2.5 JAK-2 inhibition antagonizes GM-CSF-mediated STAT-5 activation in LAMA cells

We next reasoned that blocking GM-CSF-receptor signalling, e.g. by inhibition of a main down stream target kinase, JAK-2, may antagonize ic-p-STAT-5 formation, thereby overcoming GM-CSF-mediated NI resistance. AG490 is a previously described JAK-2 inhibitor, which is used at a concentration of 50-100µM in vitro (Meydan N. et al., 1996; Samanta AK. et al., 2006). Western blotting indicated that 100µM AG490 caused a complete inhibition of JAK-2 activation (phosphorylation) in LAMA cells (not shown). JAK-2 activation was therefore quantitated by FACS-based intracellular staining in presence and absence of 100µM AG490 plus/minus GM-CSF. Whereas GM-CSF activated JAK-2 irrespective of the presence of NI (**Figure 17A**), AG490 completely prevented JAK-2 activation. This translated into a reduction of GM-CSF-induced STAT-5 activation (reduction of ic-p-STAT-5) (**Figure 17B**), and, as expected, severely reduced potential of GM-CSF to confer IM or NI resistance.

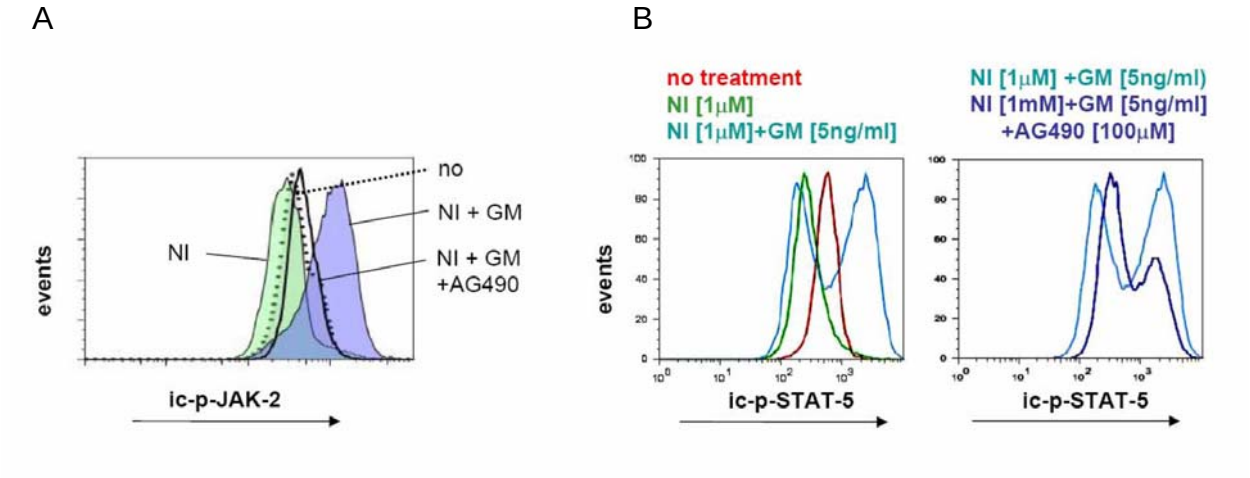


Figure 17 GM-CSF-mediated JAK-2/STAT-5 activation in LAMA cells

A. FACS histograms illustrate the levels of expression of intracellular phosphorylated JAK-2 (ic-p-JAK-2). B. The quantity of ic-p-STAT-5 after treatment of parental LAMA-cells for 48h with indicated inhibitors plus/minus GM-CSF.

4.2.6 GM-CSF induced NI-resistance of primary CML-progenitors is associated with a BCR/ABL-independent activation of STAT-5

IM and DA selectively inhibit proliferation of CML-CFC in vitro (Copland M. et al., 2006; Holtz MS. et al., 2002; Deininger MW. et al., 1997). Initial experiments with R-CM suggested that primary CML progenitors were protected from IM-induced growth inhibition due to increased concentrations of GM-CSF in 25R-CM (**Figure 13B**). GM-CSF was therefore directly tested in colony assays for its ability to mediate NI resistance of primary $CD34^+$-enriched CML progenitors derived from four consecutive CML patients in chronic phase. NI at 10µM achieved a maximum growth inhibition in this assay and was therefore used as standard concentration in colony formation experiments. GM-CSF still potently protected from NI-induced growth inhibition of BCR/ABL positive primary CML precursors (**Figure 18A**). Interestingly, GM-CSF was obviously more effective than interleukin-3 (IL-3) or G-CSF in overcoming NI-mediated growth inhibition (**Figure 18A**). Both cytokines were tested in parallel, because they have previously been reported to cause abnormal progenitor cell proliferation in CML (Jiang X. et al., 1999).

We next analysed the GM-CSF-associated signal transduction events that were associated with NI resistance in primary progenitors. Gating for $CD34^+$/ $CD116^{high}$ vs. $CD116^{low}$ expression established that STAT-5 activation by GM-CSF depended on the expression of the GM-CSF-receptor (CD116). The ic-p-STAT-5 levels increased exclusively in the $CD116^{high}$ (R2), but not in the $CD116^{low}$ (R1) population (**Figure 18B**). Notably, GM-CSF-induced ic-p-STAT-5 formation was not inhibited by NI (**Figure 18B** and **Figure 20**), but only the JAK-2 inhibitor AG490 (**Figure 18B** and **Figure 20**). In turn, NI effectively blocked BCR/ABL-kinase activation as documented by a GM-CSF- and CD116 expression-independent down-regulation of ic-p-CrkL (**Figure 18B** and **Figure 20**). Apparently, the BCR/ABL-independent STAT-5 activation translated into a significant increase in CFC numbers in presence of NI (**Figure 21**). This result was confirmed in another patient sample (pat. #3)(**Figure 18C**). Inhibition of JAK-2 by AG490 reduced the levels of ic-p-STAT-5 (**Figure 18B** and **Figure 20**) and abolished the protective effect of GM-CSF against NI treatment (**Figure 18C**). Thus, BCR/ABL-independent activation of STAT-5 by GM-CSF contributes to BCR/ABL-independent NI resistance in primary CML progenitors. Blocking JAK-2 may antagonize this type of resistance.

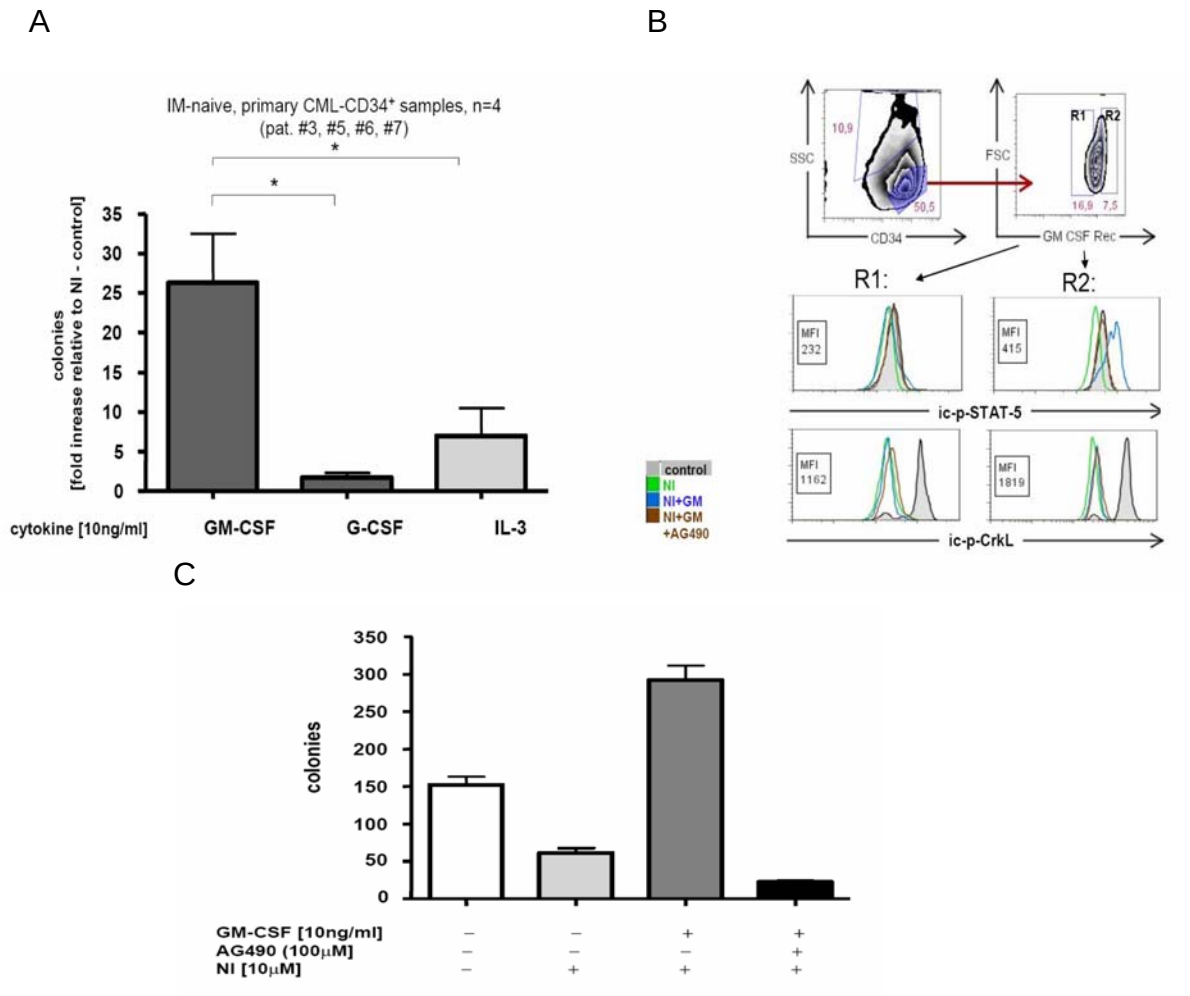


Figure 18 GM-CSF overcomes nilotinib-induced proliferation inhibition of primary CD34 positive progenitors by JAK-2/STAT-5 pathway activation

A. $CD34^+$ enriched progenitors from first diagnosis CML patients (n=4) were cultured in vitro in IMDM medium (+ 5 growth factors) in presence of [10µM] NI plus [10ng/ml] of either one of the three test cytokines (GM-CSF, IL-3 or G-CSF) as indicated. After 72h, cells were seeded in soft agar, and 10-14 days later, emerging colonies (CFC) were counted. Bars represent mean fold increase ± s.d. in CFC-counts obtained after NI exposure in presence of indicate test cytokines versus a standard cytokine cocktail only; * $p< 0.05$ (one-way ANOVA Dunnett´s adjustment for multiple comparisons). B. BCR/ABL-independent STAT-5 activation by GM-CSF in primary CML progenitors. $CD34^+$ -enriched primary CML-progenitors of patient # 7 (CML at diagnosis) were treated for 48h with NI [4µM], the JAK-2 inhibitor AG490 [100µM] plus/minus GM-CSF [10ng/ml] and analyzed by FACS for the regulation of ic-p-STAT-5 (upper two histograms) and ic-p-CrkL (lower histograms) according to the indicated gating strategy: CD34high/SSC low cells were gated into CD34 high / $CD116^{low}$ (gate R1) or $CD116^{high}$(gate R2) and separately analyzed for R1 and R2. The grey curves in each histogram plot represent baseline expression levels, the colored curves represent different

treatments as indicated. C. $CD34^+$ -enriched progenitors from a first diagnosis CML patient (pat. #3) were cultured in vitro in a liquid culture containing a cytokine cocktail of 5 growth factors (± GM-CSF, as indicated) in presence or not of NI and/or AG490. After 72h, cells were seeded in triplicates in soft agar and emerging colonies (CFC) were counted 10-14 days later. Bars represent mean CFC counts ± s.d of triplicates.

4.2.7 Overexpression of GM-CSF in IM-resistant patients

We then asked whether GM-CSF may also contribute to IM-resistance development in vivo. We reasoned that if adaptive autocrine GM-CSF secretion would be a relevant resistance/persistence mechanism in vivo via providing a proliferation or survival advantage GM-CSF-overexpressing clones would grow out during manifest IM-resistance. Indeed, when comparing GM-CSF mRNA expression in the peripheral blood (n=24), bone marrow (n=17) or peripheral blood mononuclear cells (PBMC) (n=11) of untreated chronic phase CML patients with peripheral blood samples of 29 IM-resistant patients, GM-CSF mRNA was substantially over-expressed in five of 29 IM-resistant patients (17%) (**Figure 19A, Table 2**). Increased GM-CSF was independent of the kinase mutation status and was also not related to the phase of disease at the time of resistance (**Figure 19B, Table 2**). GM-CSF overexpression at the time of IM-resistance was even more obvious when GM-CSF protein levels were studied. Elevated amounts of GM-CSF were detected in more than half of the IM-resistant patients, p= 0.007 (**Figure 19B, Table 3**). Notably, CM derived from mononuclear cells of patient #1 (patient marked with $^{\#}$ in **Figure 19A** and **B**) (**Table 1** and **2**) caused an even eight times greater expansion of IM-naïve, $CD34^+$ CML CFC in presence of NI than 25R-CM (**Figure 13B**).

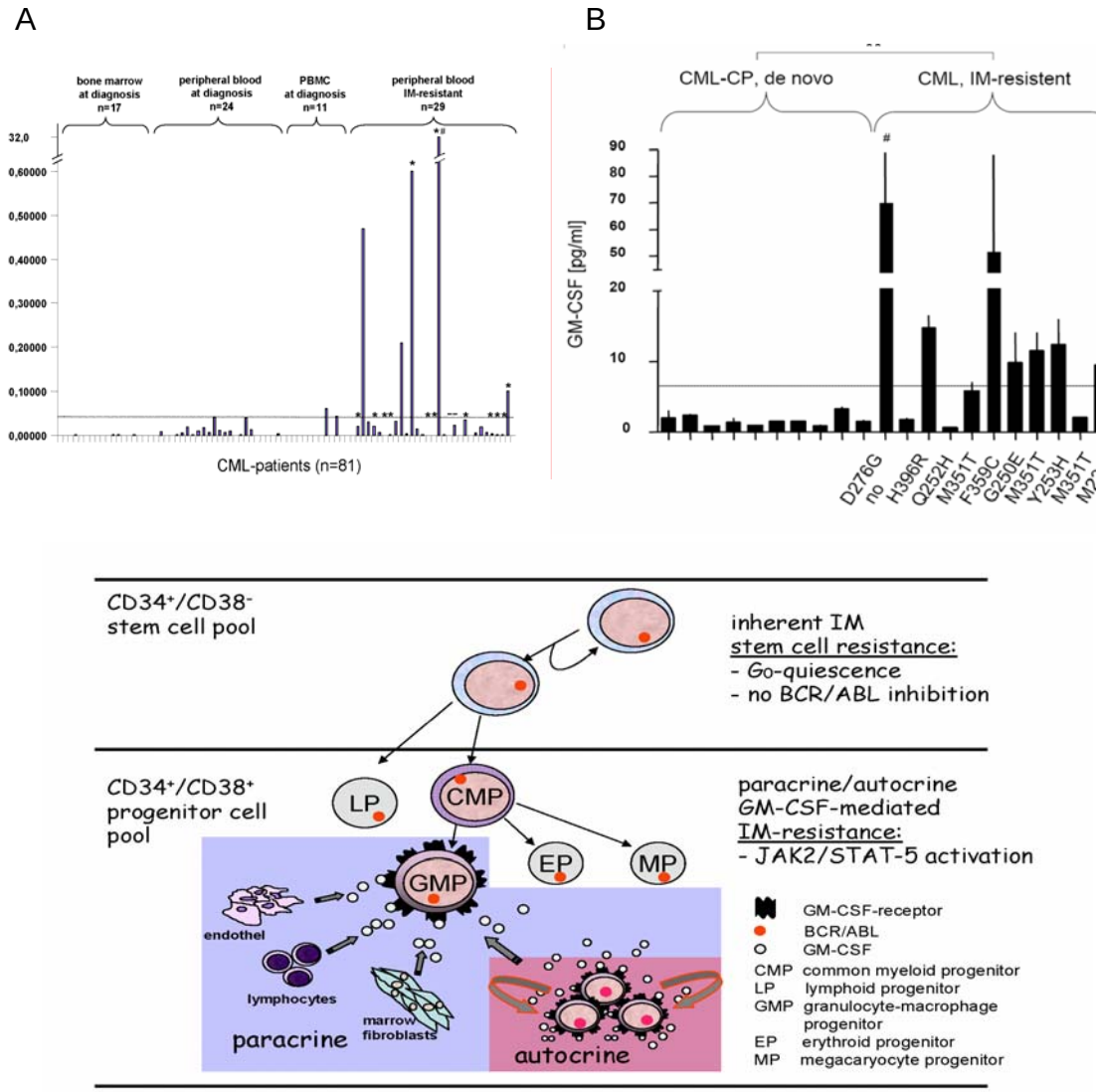


Figure 19 GM-CSF expression in primary CML samples

A. GM-CSF mRNA expression was quantitated relative to GAPDH-expression in 52 chronic phase CML patients at diagnosis and 29 CML patients at the time of clinically manifest IM-resistance. The cell sources of mRNA (bone marrow, peripheral blood or peripheral blood mononuclear cells, PBMC) are indicated; * highlights presence of a BCR/ABL-kinase mutation at the time of IM-resistance; − indicates no available kinase mutation analysis, # denotes pat. #1(PBMC). B. GM-CSF-ELISA. The GM-CSF concentration was determined in whole peripheral blood cell lysates of IM-sensitive first diagnosis CML patients and IM-resistant CML-patients. Bars represent means of duplicate measurements ± s.d.; ** $p = .006$ (Mann Whitney test). BCR/ABL-kinase mutations are shown; # designates patient #1 (see Table 1). C. Different mechanisms of NI-resistance in the progenitor and stem cell compartment. Autocrine or paracrine secretion of GM-CSF stimulates BCR/ABL-independent growth and survival of granulocyte-macrophage progenitors (GMP).

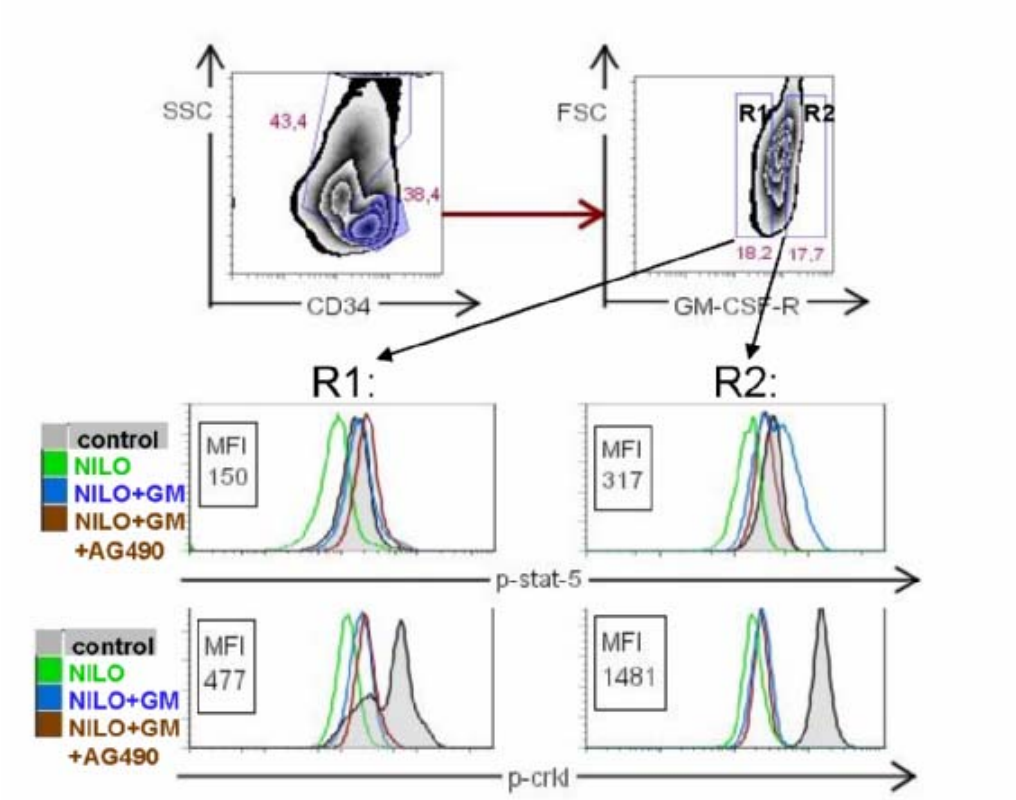


Figure 20 NILO-independent, JAK-2 dependent STAT-5 activation by GM-CSF in IM-naïve $CD34^+$ CML progenitors

$CD34^+$-enriched primary CML-progenitors of patient # 6 (de novo CML) were treated for 48h with NILO [4µM], the JAK-2 inhibitor AG490 [100µM] plus/minus GM-CSF [10ng/ml] and analyzed by FACS for the regulation of ic-p-STAT-5 (upper two histograms) and ic-p-CrkL (lower histograms) according to the gating strategy: CD34 high / $CD116^{low}$ (gate R1) or $CD116^{high}$ (gate R2). The grey curves in each histogram represent baseline expression levels.

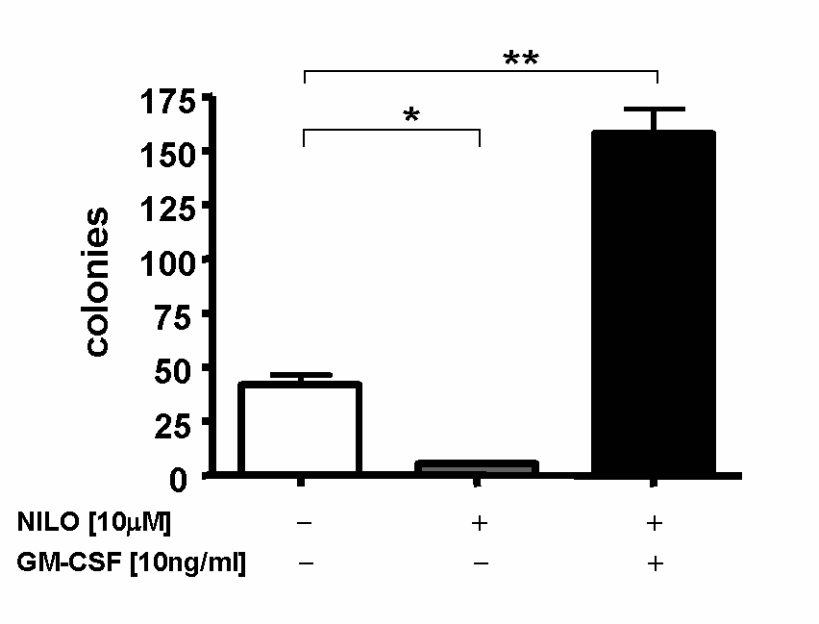


Figure 21 GM-CSF overcomes NILO-mediated inhibition of colony formation of IM-naïve $CD34^+$ CML progenitors

$CD34^+$ enriched progenitors from a first diagnosis CML patients (patient # 7) were cultured in vitro in presence of 10μM NILO in a standard medium containing a cytokine cocktail of 4 growth factors plus/minus GM-CSF, as indicated. After three days, cells were seeded into soft agar, and 10-14 days later, emerging colonies (CFC) were counted. Bars show CFC-counts (colonies) after the respective treatment; * $p < 0.05$, ** $p < 0.01$ (one way ANOVA, Dunnetts adjustment for multiple testing).

#	Gender	CML phase	cells	pretreatment	Ph-status**/ BCR-ABL/ABL mRNA-ratio*	clinical IM-response at sampling
1	female	BC	PBMC, 60% blasts	HU, IFNα, Imatinib	54%*	resistant
2	female	AP	PBMC	HU, IFNα, Imatinib	84%**	resistant
3	male	CP	CD34+ cells#	no	100%**	sensitive
4	female	CP	CD34+ cells#	no	100%**	sensitive
5	female	CP	CD34+ cells#	no	100%**	sensitive
6	male	CP	CD34+ cells#	no	100%**	sensitive
7	male	CP	CD34+ cells#	no	100%**	sensitive
8	male	CP	CD34+ cells#	no	100%**	sensitive

Table 1. Patient characteristics

Abbreviations: AP, accelerated phase; BC; blast crisis; HU, hydroxyurea; PBMC, peripheral blood mononuclear cells; #, CD34 cells were enriched by magnetic bead selection

#	gender	CML-phase	IM-treatment response	therapy	BCR/ABL-kinase mutation
9	m	CP	resistant	pre DA	G250E
2	f	CP	intolerant	pre DA	no
10	f	CP	resistant	pre DA	no
11	f	CP	resistant	pre DA	H396R
12	m	CP	resistant	pre DA	no, polym. E499E
13	m	CP	resistant	pre DA	M244V
14	f	CP	resistant	pre DA	H396R
15	f	CP	resistant	pre DA	no, polym. E499E
16	m	CP	resistant	pre DA	no
17	m	AP/BC	resistant	pre DA	no
18	f	AP/BC	resistant	pre DA	G250E
19	f	AP/BC	resistant	pre DA	no
20	f	AP/BC	resistant	pre DA	no
21	f	AP/BC	resistant	pre DA	F359C, E459K
22	m	AP/BC	resistant	pre DA	E355G
1	f	AP/BC	resistant	pre DA	D276G
23	m	AP/BC	resistant	pre DA	no
24	m	AP/BC	resistant	n.a	n.a
25	m	AP/BC	resistant	pre DA	not assessed
26	f	BC	resistant	pre DA	no
27	m	BC	resistant	pre DA	G250E, F359V
28	m	BC	resistant	pre DA	no
29	m	BC	resistant	pre NI	no
30	m	AP	resistant	pre DA	no
31	f	AP	resistant	pre DA	no
32	m	AP	resistant	pre DA	E255K
33	f	AP	resistant	pre NI	Y253H
34	m	AP	resistant	pre DA	T315I
35	m	CP	resistant	pre NI	H396R

Table 2. Patient characteristics of IM-resistant patients for GM-CSF PCR

Abbreviations: AP/BC, accelerated phase/blast crisis; DA, dasatinib; polym., polymorphism; n.a., not available; f, female; m, male

#	Ph-status**/ BCR-ABL/ABL mRNA-ratio*	BCR/ABL mutation	months imatinib therapy
1	54*	D276G	24
2	84**	no	18
35	100**	H396R	16
36	100*	Q252H	0
37	100*	M351T	26
38	62*	F359C	19
39	42*	G250E	31
40	49*	M351T	26
41	100*	Y253H	7
42	49*	M351T	33
43	40*	M351T	42

Table 3. Patient characteristics of IM-resistant patients for GM-CSF ELISA

5. DISCUSSION

5.1 Compensatory PI3-kinase/Akt/mTor activation regulates imatinib resistance development

We have here demonstrated three principal findings. First, it is shown that the Akt/mTor-signaling is activated in response to IM-exposure of IM-naïve cells. Secondly, IM-induced Akt activation is critically involved in mediating early IM-resistance and inhibition of mTor prevents IM-induced Akt-activation and IM-resistance development. Finally, a heterogeneous Akt-signaling-cascade activation is demonstrated during manifest IM-resistance independently from BCR/ABL-kinase mutations. This may have implications for the responsiveness to mTor-inhibitor treatment. Our findings suggest distinct roles for Akt-signaling activation during incipient and manifest IM-resistance.

BCR/ABL-positive minimal residual disease (MRD) can be detected by PCR in most of the IM-treated CML- and Ph^+-ALL patients in complete cytogenetic remission (Hughes TP. et al., 2003; Graham SM. et al., 2002; Muller MC. et al., 2003; Scheuring UJ. et al., 2003) and provides evidence for persistence of BCR/ABL-positive cells in presence of IM, which is mechanistically poorly understood. Such MRD can not well be explained by BCR/ABL-kinase mutations. It has been suggested that stem cells may escape IM-mediated apoptosis due to the inability of IM to attack quiescent stem cells (Graham SM. et al., 2002) or by IM-drug efflux mechanism (Burger H. et al., 2004; Thomas J. et al., 2004).

In an attempt to address the question how BCR/ABL-positive cells can survive IM-treatment in the absence of kinase mutations, we here used clonal populations of BCR/ABL positive cells to induce IM-resistance. During IM-resistance induction, cell aliquots were prospectively collected at various time points allowing later genetic and biological analysis. This methodology led to the hypothesis that activation of PI3K/Akt-signaling mediates survival under IM-challenge and precedes the emergence of strong IM-resistance (**Figure 8D** and **9**). The validity and significance of this working hypothesis was confirmed by several means. First, IM-induced Akt-activation was recapitulated using the same original IM-naïve starting populations of clones 10 and 14 (**Figure 10A**). Secondly, IM elicited a time dependent Akt-/m-Tor activation also in primary BCR/ABL-positive cells in vitro (**Figure 10C**) and even in vivo (**Figure 10D**). In fact, according to the data shown in **Figure 10**, Akt-pathway activation can be detected in IM-sensitive patients in vivo in presence of IM and provides a survival signal for BCR/ABL-positive cells exposed to IM.

Akt-signal activation in response to BCR/ABL inhibition by IM may appear illogical, because BCR/ABL is upstream of Akt. On the other hand, the findings are mechanistically reminiscent to recently published data, describing an activation of the Ras/MAPK-signaling pathway in presence of IM (Kirschner KM. et al., 2003; Ohmine K. et al., 2003; Parmar S. et al., 2004; Chu S. et al., 2004). Chu et al (Chu S. et al., 2004) additionally reported in their study that the Akt-activity was unexpectedly not inhibited by IM-treatment of primary CML-cells. In view of our results, their observation could also be interpreted as a compensatory Akt-activation after BCR/ABL inhibition, resulting in a maintained Akt activation status. Thus, evidence is accumulating that incipient IM-resistance may result from recruitment of signal activity downstream of BCR/ABL, which confers survival after inhibition of BCR/ABL. Both, Akt and p70S6K can receive signal input through other kinases and proteins such as PDK-1 (Rintelen F., 2001; Pullen N., 1998), Src, the insulin receptor substrate (IRS)-1 and Ras (Kharas MG. et al., 2005). However, members of the Akt-signaling cascade are apparently also variably and autonomously activated in IM-resistant patients with BCR/ABL kinase mutations (**Figure 12A**). It is thus tempting to speculate, that a hyper-activation of Akt or mTor in individual patients contributes independently from BCR/ABL´s activation status to the biology of IM-resistance. For example, activation of the PI3K/mTor signaling increased the formation of reactive oxygen species thereby contributing to BCR/ABL-transformation, but mTor inhibition antagonized this (Kim JH. et al., 2005). Thus, selective mTor pathway activation may translate into a differential effectiveness of mTor-inhibitors in overcoming IM-resistance. This idea is suggested from studies with mTor-inhibitors in AML(Recher C. et al., 2005) and solid cancers (Noh WC. et al., 2004), but is also shown in two patients with IM-resistant, Ph^+-ALL in this study (**Figure 12B**). This may be relevant, because even though it is established that inhibitors of the PI3K/Akt-singnaling pathway, including Rap, or a novel PDK-inhibitor (Tseng PH. et al., 2005) synergize with IM to mediate apoptosis of BCR/ABL-positive cells (Skorski T. et al., 1995; Ly C. et al., 2003; Mohi MG. et al., 2004; Tseng PH. et al., 2005), determinants for responsiveness to PI3K/Akt/mTor-inhibitors of IM-resistant disease in vivo are unknown. A third line of support that the PI3K/Akt-signaling is particularly important to mediate survival in response to IM-challenge came from mTor-inhibitor studies, where Rap and RAD001 significantly retarded or potently prevented de novo IM-resistance development in vitro (**Figure 10B, Figure 11**). These positive results are not unexpected (Skorski T. et al., 1995; Ly C. et al., 2003; Mohi MG. et al., 2004; Tseng PH. et al., 2005). However, our work extends previous findings by showing that the synergism between

Rap/RAD001 and IM in preventing IM-resistance (**Figure 11**) may be particularly due to the prevention of an IM-induced activation of Akt-signaling by mTor inhibitors (**Figure 10**). IM-induced PI3K/Akt/m-Tor-activation could contribute to a persistence of BCR/ABL-positive cells in CML patients and thus facilitate the evolution of strong, BCR/ABL-dependent IM-resistance. Notably, it has been shown that the PI3K/Akt pathway activation is also important in mediating resistance to another kinase inhibitor, gefitinib, in lung cancer (Engelman JA. et al., 2005). Use of mTor-inhibitors in combination with IM appears to be particularly suitable before strong, BCR/ABL-dependent resistance occurs.

5.2 Adaptive secretion of the Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) mediates BCR/ABL-positive progenitors resistance to Imatinib and Nilotinib via JAK-2/STAT-5 pathway activation

The rational development and successful clinical application of second generation ABL-inhibitors such as NI and DA demonstrates that the understanding of molecular mechanisms of IM resistance is of decisive clinical value (Kantarjian H. et al. et al., 2006; Talpaz M. et al., 2006). However, disease persistence and its significance for the development of outright clinical resistance is still little understood. Primary CML CD34+ progenitor cells are growth inhibited but supposedly not killed in vitro by IM (Deininger MW. et al., 1997; Holtz MS. et al., 2002; Graham SM. et al., 2002) or DA (Copland M. et al., 2006) in vitro. This may be due to an inherent inhibitor resistance of quiescent progenitors and BCR/ABL overexpression (Copland M. et al., 2006). Kinase mutations (Chu S. et al., 2005), drug in-and efflux mechanisms leading to low intracellular concentrations of IM may also contribute to persistence (Thomas J. et al., 2004; Jordanides NE. et al., 2006). Together, most IM resistance and persistence models are in line with a concept of BCR/ABL-dependent survival. Here we have identified that GM-CSF, as a hematopoietic cytokine, conferred BCR/ABL-independent NI and IM resistance by activating JAK-2/STAT-5 and thus bypassing BCR/ABL inhibition.

Aberrant production of haematopoietic cytokines, particularly of IL-3, but also G-CSF and GM-CSF, has long been suggested to play a role in CML biology (Hariharan IK. et al., 1988; Jiang X. et al., 1999; Zhang X. et al., 1998). IL-3 and GM-CSF were initially reported to cause autocrine growth loops in certain BCR/ABL positive cell lines (Hariharan IK. et al., 1988; Sirard C. et al., 1994). In addition, mice, transplanted with BCR/ABL-transduced bone marrow showed elevated IL-3 and GM-CSF levels (Zhang X. et al., 1998), and GM-CSF levels were also found to be

elevated in the serum of CML patients (Balleari E. et al., 1994). Strong evidence was then provided for IL-3 and G-CSF to mediate an autocrine growth loop in primary human CML CD34+ cells, leading to activation of STAT-5 (Jiang X. et al., 1999). These data altogether established that autocrine stimulation via G-CSF, IL-3 and GM-CSF is of pathophysiological relevance in CML by regulating progenitor cell growth and survival.

We describe here a novel role for auto/paracrine GM-CSF secretion as a counter regulatory mechanism of BCR/ABL positive cells to overcome IM/NI-induced apoptosis and proliferation inhibition. In different cell systems we showed that the GM-CSF-mediated JAK-2/STAT-5 pathway activation circumvents the need for BCR/ABL–signaling to maintain survival or proliferation, and that the leukemic population itself activates JAK-2 signaling via auto/paracrine secretion of GM-CSF. Interestingly, only GM-CSF and to a lesser extend also IL-3, but none of the other tested cytokines (MCP-1, IL-6, IL-8, VEGF, G-CSF) overcome NI-mediated CFC-inhibition (**Figure 18 A**). Both, GM-CSF and IL-3, have in common that they are strong inducers of JAK-2/STAT-5, which are critical antiapoptotic and transforming targets of BCR/ABL (Xie S. et al., 2001; Horita M. et al., 2000).

The function of GM-CSF is strictly CD116 expression-dependent (**Figure 16, Figure 18B**). GM-progenitors(GMP) express CD116 and are biologically particularly interesting as protected targets by GM-CSF, because they supposedly constitute a progenitor population, which, after acquisition of respective mutations, constitute the leukemic, self renewing stem cell population in CML blast crisis(**Figure 19**)(Jamieson CH. et al., 2004).

The mechanism of adaptive GM-CSF overexpression in response to IM-exposure is unclear at present. However, it is known that expression changes are easier to achieve by tumor cells than strategic genetic alterations such as de novo BCR/ABL kinase mutations (Hanahan D. et al., 2000). Thus, GM-CSF overexpression/secretion in response to the IM-selection pressure may even precede kinse mutation development, e.g. also in persisting clones. At the time of manifest IM-resistance, GM-CSF may even become dispensable as a resistance factor and its expression may be switched off. This is at least seen in some of the LAMA-cell clones that we have studied for GM-CSF expression in a longitudinal manner (Burchert A. et al., 2005). Lack of GM-CSF overexpression at the time of outright IM resistance in vivo, e.g., in the presence of kinase mutations would reflect this in vitro observation (**Figure 19A**).

Since GM-CSF is not only secreted by CML cells (before (Balleari E. et al., 1994) and in response to IM exposure), but also by BCR/ABL-negative cell types (**Figure 19C**), progenitor clones that do

not themselves upregulate GM-CSF could be protected by paracrine stimulation. In fact, we found evidence for STAT-5 activation of LAMA cells also when testing conditioned media collected ex vivo from the peripheral blood mononuclear samples of CML-patients in complete remission, indicating that paracrine secretion from BCR/ABL negative cells could be a relevant source for GM-CSF at the time of complete remission (not shown). Thus, GM-CSF-irrespective of what cellular source-can protect primary CML progenitors from IM- and NI-induced proliferation inhibition.

Our data have also strong clinical implications as JAK-2 inhibition could overcome GM-CSF-mediated STAT-5 activation, IM and NI resistance. This also implies that a combined BCR/ABL and JAK-2 inhibition may be a more effective future CML treatment even in IM-sensitive patients, leading to deeper molecular responses, the prevention of kinase mutation development, and consequently a reduced risk for disease progression. Interestingly, this supports nicely previous and very recent evidence showing that JAK-2 is a critical target in CML (Xie S. et al., 2001; Samanta AK. et al., 2006).

6. SUMMARY

Molecular targeted therapy with imatinib against BCR/ABL, a unique genetic abnormality of chronic phase CML, has been concluded a revolutionary improvement in the therapy of leukaemia. However, occurrence of imatinib-resistance often leads to clinical relapse in a high percentage of advanced phases of CML and Ph^+ ALL, as well as minority of CML with chronic phase. Until now ABL kinase mutations and overexpression of BCR/ABL are known to be major mediators of clinical resistance. Strategies overcoming imatinib-resistance with dose escalation, novel alternatives of imatinib, and combinations between imatinib and specific involving pathway inhibitors have shown some promising results but are far from satisfactory. Therefore, further investigating more imatinib-resistant mechanisms is necessary to provide new rationale to overcome drug resistance.

As stated above, ABL kinase mutations and overexpression of BCR/ABL contribute to overt clinical resistance. It is still a mystery how imatinib-naïve cells survive attacking of killing dose of imatinib at early treatment. We therefore made much effort to follow the reactions of 29 subclones of BCR/ABL positive, imatinib-naïve LAMA84 to increasing concentrations of imatinib in vitro. Finally, total 19 subclones reached resistance to imatinib at 1uM. In retrospect, we analyzed BCR/ABL signaling transduction pathways of several subclones at different time points before they obtained potential drug resistance. We found that a compensatory activation of PI3/Akt/mTor in imatinib-naïve LAMA84 cells may contribute early drug “resistance”. In agreement with this hypothesis, inhibition of PI3K/Akt/mTor signaling pathway respectively with wortmannin, SH6, Akt specific siRNA or mTor inhibitors dramatically restored the sensitivity of the “resistant” subclones to corresponding concentrations of IM and retarded development of imatinib-resistance in LAMA84 subclones and CML primary cells in vitro. Together, a BCR/ABL-independent and compensatory activation of PI3K/Akt/mTor pathway may represent a novel refractory mechanism for leukaemia cells to survive imatinib in vivo. Thus, blocking PI3K/Akt/mTor signaling pathway may be helpful to prevent or retard development of imatinib resistance in vivo.

In vitro it has been showed that exogenous IL-3 and EPO could antagonize apoptotic induction by imatinib in two cells model systems, indicating cytokines could also be involved in imatinib resistance. On the other side, physiological normal hematopoiesis and pathological leukamogenesis are closely associated with cytokines in auto/paracrine modes. In an attempt to address whether auto/paracrine mechanism contributes to IM-resistance in our cell model,

conditioned media generated from previously constructed IM-resistant subclones and leukemia cells of imatinib-refractory patients potentially protected parental LAMA84 and $CD34^+$ leukemia cells from apoptosis and CFC formation inhibition by imatinib and nilotinib respectively, but not unresistant mock controls, indicating that a strong IM- and NI-resistance existed in conditioned media (CM). A cytokine array was applied and several cytokines were identified to be produced by imatinib-refractory LAMA84 subclones, compared with unresistant control cells. Among them, only granulocyte-macrophage colony stimulating factor (GM-CSF) could repeat the phenotype of CM originated from IM-resistant subclones. Neutralising antibody against GM-CSF abrogated the protective role of CM. Clinically, secreted GM-CSF in CML IM-resistant patients also supported this finding. Moreover, GM-CSF exclusively protect GM-CSF receptor (CD116) highly expressed progenitors from IM and Ni-induced apoptosis by activating STAT5. The mechanism of imatinib and nilotinib resistance conferred by GM-CSF is due to a BCR/ABL independent activation of JAK2/STAT5 signaling pathway. Blocking JAK2 pathway with AG490 decreased STAT5 phosphorylation and overcame GM-CSF mediated imatinib and nilotinib resistance. Therefore here we introduce a new mechanism of resistance namely that GM-CSF induced JAK2/STAT5 activation could substitutes for BCR/ABL-signaling to maintain growth and survival of BCR/ABL-positive leukemic progenitors in presence of imatinib or nilotinib. Simultaneous inhibition of JAK2/STAT5 pathway and BCR/ABL could be a potential alternative therapy for IM-resistance.

7. REFERENCES

Al-Ali HK, Heinrich MC, Lange T, et al. High incidence of BCR-ABL kinase domain mutations and absence of mutations of the PDGFR and KIT activiation loops in CML patients with secondary resistance to imatinb. Hematol J 2004; 5:55-60.

Andoniou CE, Thien CBF, Langdon WY. The two major sites of cbl tyrosine phosphorylation in abl-transformed cells select the crkL SH2 domain. Oncogene 1996; 12:1981-89

Baccarani M. Hematology-the European Hematology Association Education Program 2006; 2:(1).

Bhatia R, Holtz MS, Niu N, et al. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. Blood 2003; 101:4701-4707.

Balleari E, Bason C, Visani G, et al. Serum levels of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor in treated patients with chronic myelogenous leukemia in chronic phase. Haematologica 1994; 79:7-12.

Bartram CR, de Klein A, Hagemeijer A, et al. Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chonic myelocytic leukemia. Nature 1983; 306:277-80.

Ben-Neriah Y, Daley GQ, Mes-Masson A-M, et al. The chronic myelogenous leukemia-specific p210 protein is the product of the bcr/abl hybrid gene. Science 1986; 233:212-14.

Bennett J. Case of hypertrophy of the spleen liver, in which death took place from suppuration of the blood. Edinb Med Surg J 1845; 64:413-23.

Beran M, Cao X, Estrov Z, et al. Selective inhibition of cell proliferation and BCR-ABL phosphorylation in acute lymphoblastic leukaemia cells expressing Mr 190,000BCR-ABL protein by a tyrosine kinase inhibitor(CGP-57148). Clin Cancer Res 1998; 4:1661-72.

Bhatia R, Holtz M, Niu N, et al. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. Blood 2003; 101:4701-7.

Bonifazi F, de Vivo A, Rosti G, et al. Chronic myeloid leukemia and interferon-alpha: a study of complete cytogenetic responders. Blood 2001; 98(10):3074-81.

Branford S, Rudzki Z, Walsh S, et al. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. Blood

2002; 99:3472-5.

Branford S, Rudzki Z, Walsh S, et al. High frequency of point mutations clustered within the adenosine triphosphate binding region of BCR/ABL in patients with chronic myeloid leukaemia or Ph-positive acute lymphoblastic leukaemia who develop imatinib(STI571) resistance. Blood 2002; 99:3472-3475.

Branford S, Rudzki Z, Walsh S, et al. Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. Blood 2003; 102:276-83.

Buchdunger E, Zimmermann J, Mett H, et al. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. Cancer Res 1996; 56:100-4.

Buchdunger E, Cioffi CL, Law N, et al. Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. J Pharmacol Exp Ther 2000; 295:139-45.

Bueno-da-Silva AE, Brumatti G, Russo FO, et al. Bcr/Abl-mediated resistance to apoptosis is independent of constant tyrosine- kinase activity. Cell Death Differ 2003; 10:592-598.

Burchert A, Wang Y, Cai D, et al. Compensatory PI3-kinase/Akt/mTor activation regulates imatinib resistance development. Leukemia 2005; 19:1774-82.

Burger H, van Tol H, Boersma AW, et al. Imatinib mesylate(STI) is substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump. Blood 2004; 104:2940-42.

Cai D, Wang Y, Ottomann OG, et al. FLT3-ITD, but not BCR/ABL-transformed cells require concurrent Akt/mTor blockage to undergo apoptosis after histone deacetylase inhibitor treatment. Blood 2006; 107:2094-97.

Canaani E, Steiner-Saltz D, Aghai E, et al. Altered transcription of an oncogene in chronic myeloid leukemia. Lancet 1984; 1:593-95.

Carpino N, Wisniewski D, Strife A, et al. $p62^{dok}$: A constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. Cell 1997; 88:197-204.

Carroll M, Ohno-Jones S, Tamura S, et al. CGP 57148, a tyrosine kinase inhibitor, inhibits the growth of cells expressing BCR-ABL, TEL-ABL, and TEL-PDGFR fusion proteins. Blood 1997; 90:4947-52.

Chu S, Holt M, Gupta M, Bhatia R. BCR/ABL kinase inhibition by imatinib mesylate enhances

MAP kinase activity in chronic myelogenous leukemia CD34+ cells. Blood 2004; 103:3167-74.

Chu S, Xu H, Shah NP, et al. Detection of BCR-ABL kinase mutations in CD34+ cells from chronic myelogenous leukemia patients in complete cytogenetic remission on imatinib mesylate treatment. Blood 2005; 105:2093-8.

Clark SC, McLaughlin J, Timmons M, et al. Expression of a distinctive BCR-ABL oncogene in Ph1-positive acute lymphocytic leukemia(ALL). Science 1988; 239:775-77.

Coiffier B, Lepage E, Brière J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med 2002; 346:235-242.

Copland M, Hamilton A, Elrick LJ, et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML, but does not eliminate the quiescent fraction. Blood 2006; 107(11):4532-9.

Corbin AS, Rosee PL, Stoffregen EP, et al. Several Bcr/Abl kinase domain mutants associated with imatinib mesylate resistance remain sensitive to imatinib. Blood 2003; 101:4611-14.

Cortez D, Reuther G, Pendergast AM. The Bcr-Abl tyrosine kinase activates mitogenic signaling pathways stimulates G1-to-S phase transition in hematopoietic cells. Oncogene 1997; 15:2333-42.

Craigie D. Case of disease of the spleen, in which death took place in consequence of the presence of purulent matter in the blood. Edinb Med Surg J 1845; 64:400-13.

Dai Y, Rahmani M, Pei XY, et al. Bortezomib and flavopiridol interact synergistically to induce apoptosis in chronic myeloid leukaemia cells resistant to imatinib mesylate through both Bcr/Abl-dependent and –independent mechanisms. Blood 2004; 104(2):509-18.

Daley GQ, Van Etten RA, Baltimore D. Introduction of chronic myelogenous leukaemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. Science 1990; 247:824-30.

Daley GQ. Towards combination target-directed chemotherapy for chronic myeloid leukemia: role of farnesyl transferase inhibitors. Semin Hematol 2003; 40(2 Suppl 2):11-4.

de Klein A, van Kessel AG, Grosveld G. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. Nature 1982; 300:765-67.

de The H, Lavau C, Marchio A, et al. The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR.

Cell 1991; 66(4):675-84.

Deininger M, Goldman JM, Lydon NB, et al. The tyrosine kinase inhibitor CGP57148B selectively inhibits the growth of BCR-ABL positive cells. Blood 1997; 90:3691-8.

Dengler J, von Bubnoff N, Decker T, et al. Combination of imatinib with rapamycin or RAD001 acts synergistically only in Bcr-Abl-positive cells with moderate resistance to imatinib. Leukemia 2005; 19(10):1835-8.

Donahue RE, Wang EA, Stone DK, et al. Stimulation of haematopoiesis in primates by continuous infusion of recombinant human GM-CSF. Nature 1986; 21:872-5.

Donahue RE, Seehra J, Metzger M, et al. Human IL-3 and GM-CSF act synergistically in stimulating hematopoiesis in primates. Science 1988; 241:1820-3.

Donato NJ, Wu JY,stapley J, et al. BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukaemia cells selected for resistance to STI571. Blood 2003; 101:690-698.

Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 1996; 2:561-6.

Druker BJ, Lydon NB. Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukaemia. J Clin Invest 2000; 105:3-7.

Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCRABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 2001; 344:1031-37.

Druker BJ, Sawyers CL, Kantarjian H, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med 2001; 344:1038-42.

Druker BJ, O'Brien SG, Cortes J, Radich J. 2002. Chronic myelogenous leukemia. Hematology 2002:111-35

Eisen HJ, Tuzcu EM, Dorent R, et al. Everolimus for the prevention of allograft rejection and vasculopathy in cardiac-transplant recipients. N Eng J Med 2003; 349:847-58.

Engelman JA, Janne PA, Mermel C, et al. ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. Proc Natl Acad Sci USA 2005; 102:3788-3793.

Era T, Witte ON. Regulated expression of P210 Bcr-Abl during embryonic stem cell differentiation stimulates multipotential progenitor expansion and myeloid cell fate. Proc.Natl Acad Arts Sci 2000; 97:1737-42.

Fenaux P, Chevret S, Guerci A, et al. Long-term follow-up confirms the benefit of all-trans retinoic acid in acute promyelocytic leukemia. European APL group. Leukemia 2000; 14(8):1371-7.

Gambacorti-passerini C, le Coutre P, Mologni L, et al. Inhibition of the ABL kinase activity blocks the proliferationof BCR/ABL + leukemic cells and induces apoptosis. Blood Cell Mol Dis 1997; 23:380-394.

Gishizky ML, Johnson-White J, Witte ON. Efficient transplantation of BCR/ABL induced chronic myelogenous leukemia-like syndrome in mice. Proc Natl Acad Sci USA 1993; 90:3755-59.

Golas JM, Arndt K, Etienne C, et al. SKI-606, a 4-anilino-3-quinolinecarbonitrile dual inhibitor of Src and Abl kinases, is a potent antiproliferative agent against chronic myelogenous leukemia cells in culture and causes regression of K562 xenografts in nude mice. Cancer Res 2003; 63(2):375-81.

Golas JM, Lucas J, Etienne C, et al. SKI-606, a Src/Abl inhibitor with in vivo activity in colon tumor xenograft models. Cancer Res 2005; 65(12):5358-64.

Goldman JM, Melo JV. Targeting the BCR-ABL tyrosine kinase in chronic myeloid leukaemia. N Eng J Med. 2001 Apr 5; 344(14): 1084-6

Golub TR, Goga A, Barker GF, et al. Oligomerization of the ABL tyrosine kinase by the ETS protein TEL in human leukaemia. Mol Cell Biol 1996; 16:4107-16.

Gorre ME, Mohammed M, Ellwood K, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 2001; 293:876-80.

Gorre ME, Sawyers CL. Molecular mechanisms of resistance to STI571 in chronic myeloid leukaemia. Curr Opin Hematol. 2002; 9(4):303-7.

Graham, S.M. et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood 2002; 99:319-25.

Groffen J, Heisterkamp N, Stephenson JR, et al. c-sis istranslocated from chromosome 22 to chromosome 9 in chonic myelocytic leukaemia. J Exp Med 1983; 158:9-15.

Grosveld G, Verwoerd T, van Agthoven T, et al. The chronic myelocytic cell line K562 contains a breakpoint in bcr and products a chemeric bcr/c-abl transcript. Mol Cell Biol 1986; 6:607-16.

Gumireddy K, Baker SJ, Cosenza SC, et al. A non-ATP-competitive inhibitor of BCR-ABL overrides imatinib resistance. Proc Natl Acad Sci U S A 2005; 102(6):1992-7.

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100:57-70.

Hariharan IK, Adams JM, Cory S. bcr-abl oncogene renders myeloid cell line factor independent: potential autocrine mechanism in chronic myeloid leukemia. Oncogene Res 1988; 3:387-399.

Hehlmann R, Heimpel H, Hasford J, et al. Randomized comparison of interferon-alpha with busulfan and hydroxyurea in chronic myelogenous leukemia. The German CML Study Group. Blood. 1994; 84(12):4064-77.

Heisterkamp N, Stam K, Groffen J, et al. Structure organization of the bcr gene and ist role in the Ph' translocation. Nature 1985; 315:758-61.

Heisterkamp N, Jenster G, ten Hoeve J, et al. Acute leukaemia in bcr/abl transgenic mice. Nature 1990; 344:251-53.

Hermans A, Heisterkamp N, von Lindern M, et al. Unique fusion of bcr and c-abl gens in Philadelphia chromosome positive acute lymphoblastic leukemia. Cell 1987; 51:33-40.

Hochhaus A, Kreil S, Corbin AS, et al. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. Leukemia 2002; 16:2190-6.

Hofmann WK, de Vos S, Elashoff D, et al. Relation between resistance of Philadelphia-chromosome-positive acute lymphoblastic leukaemia to the tyrosine kinase inhibitor STI571 and gene-expression profiles: a gene-expression study. Lancet 2002; 359:481-6.

Holt MS, Slovak ML, Zhang F, et al. Imatinib mesylate(STI571) inhibits growth of primitive malignant progenitors in chronic myelogenous leukaemia through reversal of abnormally increased proliferation. Blood 2002; 99:3792-3800.

Holtz MS, Forman SJ and Bhatia R. Nonproliferating CML Cd34+ progenitors are resistant to apoptosis induced by a wide range of proapoptotic stimuli. Leukemia 2005; 19:1034-41.

Holyoake T, Allan EK, Grand F, et al. Combination therapies including imatinib do not eradicate quiescent chronic myeloid leukaemia stem cells in vitro(abstract).Blood 2003; 102:71a.

Horita M, Andreau EJ, Benito A, et al. Blockade of the Bcr-Abl kinase activity induces apoptosis of chronic myelogenous leukemia cells by suppressing signal transducer and activator of transcription 5-dependent expression of Bcl-xL. J Exp Med 2000; 191, 977-84.

Huang S. and Houghton PJ. Targeting mTOR signalling for cancer therapy. Curr Opin Pharmacol 2003; 3:371-7.

Hughes TP, Kaeda J, Branford S, et al. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukaemia. N Engl J Med 2003; 349:1423-1432.

Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004; 350:2335-2342.

Jabbour E, Kantarjian H, Jones D, et al. Frequency and clinical significance of BCR-ABL mutations in patients with chronic myeloid leukemia treated with imatinib mesylate. Leukemia. 2006; 20(10):1767-73.

Jamieson CH, Ailles LE, Dylla SJ, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med 2004; 351:657-67.

Jiang X, Lopez A, Holyoake T, et al. Autocrine production and action of IL-3 and granulocyte colony-stimulating factor in chronic myeloid leukemia. Proc Natl Acad Sci U S A 1999; 96:12804-9.

Jiang X, Zhao Y, Chan P, et al. Quantitative real-time RT-PCR analysis shows BCR-ABL expression progressively decreases as primitive leukaemic cells from patients with chronic myeloid leukaemia(CML) differentiate in vivo. Exp Hematol 2003; 31:228-9.

Jordanides NE, Jorgensen HG, Holyoake TL, et al. Functional ABCG2 is over-expressed on primary CML CD34+ cells and is inhibited by imatinib mesylate. Blood 2006; 108(4):1370-3.

Kakizuka A, Miller WH Jr, et al. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. Cell 1991; 66(4):663-74.

Kantarjian HM, Talpaz M, Hester J. et al. Collection of peripheral –blood diploid cells from chronic myelogenous leukemia patients early in the recovery phase from myelosuppression induced by intensive-dose chemotherapy. J Clin Oncol. 1995 Mar;13(3):553-9.

Kantarjian HM, O'Brien S, Smith TL, et al. Treatment of Philadelphia chromosome-positive early chronic phase chronic myelogenous leukemia with daily doses of interferon alpha and low-dose cytarabine. J Clin Oncol 1999; 17(1):284-92.

Kantarjian H, Sawyers C, Hochhaus A, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. N Engl J Med 2002; 346:645-52.

Kantarjian HM, Talpaz M, O'Brien S, et al. Dose escalation of imatinib mesylate can overcome resistance to standard-dose therapy in patients with chronic myelogenous leukaemia. Blood 2003; 101:473-5.

Kantarjian H, Talpaz M, O'Brien S, et al. High-dose imatinib mesylate therapy in newly diagnosed Philadelphia chromosome-positive chronic phase chronic myeloid leukaemia. Blood 2004; 103(8):2873-8.

Kantarjian H, Talpaz M, O'Brien S, et al. Survival benefit with imatinib mesylate therapy in patients with accelerated-phase chronic myelogenous leukemia—comparison with historic

experience. Cancer 2005; 103(10):2099-108.

Kantarjian H, Giles F, Wunderle L, et al Nilotinib in imatinib-resistanct CML and Philadelphia chromosome-positive ALL. N Engl J Med 2006; 354:2542-51.

Kharas MG, Frumann DA. ABL oncogenes and phosphoinositide 3-kinase: Mechanism of activation and downstream effectors. Cancer Res 2005; 65:2047-53.

Kirschner KM, Baltensperger K. Erythropoietin promotes resistance aginst the Abl tyrosine kinase inhibitor imatinib (STI571) in K562 human leukaemia cells. Mol Cancer Res 2003; 1:970-80.

Kim JH, Chu SC, Gramlich JL, et al. Activaon of the PI3K/mTor pathway by BCR-ABL contributes to increased production of reactive oxygen species. Blood 2005; 105:1717-23.

Kozikowski AP, Sun H, Brognard J, et al. Novel PI analoguesmselectively block activation of the prosurvival serine/threonine kinase Akt. J Am Chem Soc 2003; 125:1144-5.

La Rosee P, O'Dwyer ME, Druker BJ. Insights from pre-clinical studies for new combination treatment regimens with the Bcr-Abl kinase inhibitior imatinib mesylate (Gleevec/Glivc) in chronic myelogenous leukaemia: a translational perspective. Leukemia 2002; 16:1213-9.

Leahy MF, Seymour JF, Hicks RJ, et al. Multicenter phase II clinical study of iodine-131-rituximab radioimmunotherapy in relapsed or refractory indolent non-Hodgkin's lymphoma. J Clin Oncol 2006; 24(27):4418-25.

le Coutre P, Tassi E, Varella-Garcia M, et al. Induction of resistance to the abelson inhibitor STI571 in human leukemic cells through gene amplification. Blood 2000; 95:1758-66.

Li S, Gillessen S, Tomasson MH, et al. Interleukin 3 and granulocyte-macrophage colony-stimulating factor are not required for induction of chronic myeloid leukemia-like myeloproliferative disease in mice by BCR/ABL. Blood 200; 97(5):1442-50.

Lichtman MA. Chronic myelogenous leukemia and related disorders. In Williams Hematology, ed. E Buetler, MA Lichtman, BS Coller, TJ Kipps, 1995, pp. 298-324. New York: McGraw-Hill.

Longo L, Pandolfi PP, Biondi A, et al. Rearrangements and aberrant expression of the retinoic acid receptor alpha gene in acute promyelocytic leukemias. J Exp Med 1990; 172(6):1571-5.

LyC, Arechiga AF, Melo JV, Walsh CM, Ong ST. Bcr-Abl kinase modulates the translation regulators ribosomal protein S6 and 4E-BP1 in chronic myelogenous leukaemia cells via the mammalian target of rapamycin. Cancer Res 2003; 63:5716-22.

Mahon FX, Belloc F, Lagarde V, et al. MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. Blood 2003; 101:2368-73.

Marin D, Glodman JM, Olavarria E, et al. Transient benefit only from increasing the imatinib dose

in CML patients who do not achieve complete cytogenetic remissions on conventional doses. Blood 2003; 102:2702-3.

McWhite JR, Galasso DL, Wang JYJ. A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoprotein. Mol Cell Biol 1993; 13:7587-95.

Melo JV. The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype. Blood 1996; 88:2375-84.

Mes-Masson A-M, Mclaughin J, Daley G, et al. Overlapping cDNA clones define the complete coding region for the $P210^{+}$c-abl gene product associated with chronic myelogenousleukemia cells containing the Philadelphia chromosome. Proc Natl Acad Sci USA 1986; 83:9768-72.

Meydan N, Grunberger T, Dadi H, et al. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. Nature 1996; 379:645-8.

Mohi MG, Boulton C, Gu TL, et al. Combination of rapamycin and protein tyrocin kinase (PTK) inhibitors for the treatment of leukemias caused by oncogenic PTKs. Proc Natl Acad Sci USA 2004; 101:3130-5.

Muller MC, Gattermann N, Lahaye T, er al. Dynamics of BCR-ABL mRNA expression in first line therapy of chronic myelogenous leukemia patients with imatinib or interferon alpha/ara-C. Leukemia 2003; 17:2392-2400.

Muller AJ, Young JC, Pendergast A-M, et al. BCR first exon sequences specifically activate Bcr/Abl tyrosine kinase oncogene of Philadelphia chromosome positive human leukemias. Mol Cell Biol 1991; 11:1785-92.

Niove E, Jordanides, Heather G, et al. Functional ABCG2 is overexpressed on primary CML $CD34^{+}$ cells and is inhibited by imatinib mesylate. Blood 2006; 108(4):1370-3.

Noh WC, Mondesire WH, Peng J, et al. Determinants of rapamycin sensitity in breast cancer cells. Clin Cancer Res. 2004 Feb 1;10(3):1013-23.

Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. Science 1960; 132:1497.

O'Brien SG, Guilhot F, Larson RA, et al. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med 2003; 348:994-1004.

Oda T, Heaney C, Hagopian JR, et al. Crkl is the major tyrosine-phosphorylated protein in neutrophils from patients with chronic myelogenous leukemia. J Biol Chem 1994;

269:22925-8.

O'Hare T, Walters DK, Stoffregen EP, et al. In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 agianst clinically relevant imatinib-resistant Abl kinase domain mutants. Cancer Res 2005; 65:4500-5.

Ohmine K, Nagai T, Tarumoto T, et al. Analysis of gene expression profiles in an imatinib-resistant cell line, KCL22/SR. Stem Cells 2003; 21:315-21.

Parmar S, Katsoulidis E, Verma A, et al. Role of the p38 mitogen-activated protein kinase pathway in the generation of the effects of imatinib mesylate (STI571) in BCR-ABL-expressing cells. J Biol Chem 2004; 279:25345-352.

Pandolfi PP, Alcalay M, Fagioli M, et al. Genomic variability and alternative splicing generate multiple PML/RAR alpha transcripts that encode aberrant PML proteins and PML/RAR alpha isoforms in acute promyelocytic leukaemia. EMBO J 1992; 11(4):1397-407.

Pane F, Frigeri F, Sindona M, et al. Neutraphilic-Chronic Myeloid Leukemia: A distinct disease with a specific molecular marker(BCR/ABL with C3/A2 junction). Blood 1996; 88:2410-14.

Pendergast AM, Muller AJ, Havlik MH, et al. BCR sequences essential for transformation by the BCR/ABL oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner. Cell 1991; 66:161-71.

Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 2005; 353:1659-1672.

Press RD, Love Z, Tronnes AA, et al. BCR-ABL mRNA levels at and after the time of a complete cytogenetic response (CCR) predict the duration of CCR in imatinib mesylate-treated patients with CML. Blood. 2006; 107(11):4250-6.

Ptasznik A, Nakata Y, Kalota A, et al. Short interfering RNA (siRNA) targeting the Lyn kinase induces apoptosis in primary, and drug-resistant, BCR-ABL1(+) leukaemia cells. Nat Med 2004; 10(11):1187-9.

Ptasznik A, Urbanowska E, Chinta S, et al. Crosstalk between BCR/ABL oncoprotein and CXCR4 signaling through a Src family kinase in human leukemia cells. J Exp Med 2002; 196:667-78.

Puil L, Liu J, Gish G, et al. Bcr-Abl oncoproteins bind directly to activators of the Ras signalling pathway. EMBO J 1994; 13:764-73.

Pullen N, Dennis PB, Andjelkovic M, et al. Phosphorylation and activation of p70s6k by PDK1. Science 1998; 279:707-10.

Recher C, Beyne-Rauzy O, Demur C, Chicanne G, Dos Santos C, Mas VM et al. Anti-leukemic

activity of the rapamycin in acute myeloid leukemia. Blood 2005; 105:2527-34.

Rintelen F, Stocker H, Thomas G, Hafen E. PDK1 regulates growth through Akt and S6K in Drosophila. Proc Natl Acad Sci USA 2001; 98:15020-25.

Robert N, Leyland-Jones B, Asmar L, et al. Randomized phase III study of trastuzumab, paclitaxel, and carboplatin compared with trastuzumab and paclitaxel in women with HER-2-overexpressing metastatic breast cancer. J Clin Oncol 2006; 24(18):2786-92.

Roche-Lestienne C, Soenen-Cornu V, Grardel-Duflos N, et al. Several types of mutations of the Abl gene can be found in chronic myeloid leukemia patients resistant to STI571, and they can pre-exist to the onset of treatment. Blood 2002; 100:1014-8.

Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med 2005; 353:1673-1684.

Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and giemsa staining. Nature 1973; 243:290-3.

Salesse S, Verfaillie CM. 2002. BCR/ABL: from molecular mechanisms of leukaemia induction to treatment of chronic myelogenous leukaemia. Oncogene 21:8547-59

Samanta AK, Lin H, Sun T, et al. Janus kinase 2: a critical target in chronic myelogenous leukaemia. Cancer Res 2006; 66:6468-72.

Sattler M, Durstin MA, Frank DA, et al. The thrombopoietin receptor c-MPL activates JAK2 and TYK2 tyrosine kinases. Exp Hematol. 1995 Aug; 23(9):1040-8.

Sattler M, Mohi MG, Pride YB, et al. Critical role for Gab2 in transformation by BCR/ABL. Cancer Cell 2002; 1:479-92.

Sawyers CL, Hochhaus A, Feldman E, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. Blood 2002; 99:3530-9.

Scheuring UJ, Pfeifer H, Waamann B, et al. Serial minimal residual disease (MRD) analysis as a predictor of response duration in Philadephia-positive acute lymphoblastic leukaemia (Ph+ALL) during imatinib treatment. Leukemia 2003; 17:1700-6.

Schindler T, Bornmann W, Pellicena P, et al. Structural mechanism for STI571 inhibition of abelson tyrosine kinase. Science 2000; 289:1938-42.

Schulz J, McCleod M, Patel T, et al. Addition of bevacizumab to bolus fluorouracil and leucovorin in first-line metastatic colorectal cancer: results of a randomized phase II trial. J Clin Oncol 2005; 23(16):3697-705.

Shah NP, Nicoll JM, Nagar B, et al. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib(STI571) in chronic phase and blast crisis chronic myeloid leukaemia. Cancer Cell 2002; 2:117-25.

Shah NP, Tran C, Lee FY, et al. Overriding imatinib resistance with a novel ABL kinase inhibitor. Science 2004; 305(5682):399-401.

Shepherd P, Suffolk R, Halsey J, et al. Analysis of molecular breakpoint and m-RNA transcripts in a prospective randomized trial of interfon in chronic myeloid leukaemia: no correlation with clinic features, cytogenetic response, duration of chronic phase, or survival. Br J Haematol 1995; 89:546-54.

Shtivelman E, Lifshitz B, Gale RP, et al. Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. Nature 1985; 315:550-4.

Shuai K, Halpern J, ten Hoeve J, et al. Constitutive activation of STAT5 by the BCR-ABL oncogene in chronic myelogenous leukemia. Oncogene 1996; 13: 247-54.

Sirard C, Laneuville P, Dick JE. Expression of bcr-abl abrogates factor-dependent growth of human hematopoietic M07E cells by an autocrine mechanism. Blood 1994; 83:1575-85

Skorski T, Kanakaraj P, Nieborowska-Skorska M, et al. Phosphatidylinositol-3 kinase activity is regulated by BCR/ABL and is required for the growth of Philadelphia chromosome-positive cells. Blood 1995; 86:726-36.

Stewart MJ, Litz-Jackson S, Burgess GS, et al. Role for E2F1 in p210 BCR-ABL downstream regulation of c-myc transcription initiation. Studies in murine myeloid cells. Leukemia 1995; 9:1499-507.

Strauss SJ, Morschhauser F, Rech J, et al. Multicenter phase II trial of immunotherapy with the humanized anti-CD22 antibody, epratuzumab, in combination with rituximab, in refractory or recurrent non-Hodgkin's lymphoma. J Clin Oncol 2006; 24(24):3880-6.

Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resitant Philadelphia chromosome-positive leukemias. N Engl J Med 2006; 354:2531-41.

Thomas J, Wang L, Clark RE, et al. Active transport of imatinib into and out of cells: implications for drug resistance. Blood 2004; 104:3739-45.

Tseng PH, Lin HP, Zhu J, et al. Synergistic interactions between imatinib and the novel phosphoinositide-dependent kinase-1 inhibitor OSU-03012 in overcoming imatinib resistance. Blood 2005; 105:4021-7.

Varticovski L, Daley GQ, Jackson P, et al. Activation of phosphatidylinositol 3-kinase in cells

expressing abl oncogene variants. Mol Cell Biol 1991; 11:1107-13.

Virchow R..Weisses Blut. Froriep's Neue Notizen aus dem Gebiet der Natur.- und Heilkunde. Weimer 1845; 36:151–5.

Von Bobnoff N, schneller F, Peschel C, et al. BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukemia to STI571: a prospective study. Lancet 2002; 359:487-91.

Von Bubnoff N, Veach DR, Van Der Kuip H, et al. A cell-based screen for resistance of Bcr-Abl positive leukemia identifies the mutation pattern for PD166326, an alternative Abl kinase inhibitor. Blood 2004; 105:1652-9.

Weisberg E, Manley PW, Breitenstein W, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell 2005; 7:129-41.

Wong S, Witte ON. Modeling Philadelphia chromosome positive leukemias. Oncogene 2001; 20:5644-59.

Xie S, Wang Y, Liu J, et al. Involvement of Jak2 tyrosine phosphorylation in Bcr-Abl transformation. Oncogene 2001; 20:6188-95.

Yamaoka K, Saharinen P, Pesu M, et al. The Janus kinases (Jaks). Genome Biol 2004; (12)5:253.

Yu C, Rahmani M, Conrad D, et al. The proteasome inhibitor bortezomib interacts synergistically with histone deacetylase inhibitors to induce apoptosis in Bcr/Abl+ cells sensitive and resistant to STI571. Blood 2003; 102(10):3765-74.

Zhang X, Ren R. Bcr/Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte-macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. Blood 1998; 92:3829-40.

Zhao RC, Jiang Y, Verfaillie CM. A model of human p210(bcr/ABL)-mediated chronic myelogenous leukemia by transduction of primary normal human $CD34^+$ cells with a BCR/ABL-containing retroviral vector. Blood 2001; 97:2406-12.

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10. CURRICULUM VITAE

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Publications:

1. Brendel C, Scharenberg C, Dohse M, Robey R, Bates S, Schkla S**,** Wang Y, Wennemuth G, Burchert A, Boudriot U, and Neubauer A. Imatinib mesylate and nilotinib (AMN107) exhibit high affinity interaction with ABCG2 activity on primitive hematopoietic stem cells. Accepted by Leukemia
2. Wang Y, Cai D, Brendel C, Barett C, Erben P, et al. Adaptive secretion of the Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) mediates Imatinib- and Nilotinib-resistance in BCR/ABL-positive progenitors via JAK-2/STAT-5 pathway activation. Blood 2006 Nov 7; [Epub ahead of print]
3. Cai D**,** Wang Y, Ottmann OG, Barth PJ, Neubauer A, and Burchert A. FLT3-ITD-, but not BCR/ABL-transformed cells require concurrent Akt blockage to undergo apoptosis after histone deacetylase inhibitor treatment. Blood 2006; 107(5):2094-2097.
4. Burchert A, Wang Y, Cai D**,** von Bubnoff N., Paschka P, and et al. Compensatory PI3-kinase/Akt/mTor activation regulates imatinib resistance development. Leukemia 2005; 19(10):1774-82.
5. Zhang BX, Zhao P, Wang Y, and et al «OPHTHALMOLOGY» (Fourth Edition of Textbook in English) was published by Liaoning Science and Technology Publishing House 1999
6. Wang Y, Zhao P, Liu XJ, Zhang BX. Treatment of malignant glaucoma with vitreous punctomies. J of China Practical Ophthalmology 1999; 27(1):23-4.

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11. EHRENWÖRTLICHE ERKLÄRUNG

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel „Novel imatinib resistance mechanisms in chronic myeloid leukemia“ im Klinikum der
Philipps-Universität Zentrum Innere Medizin SP Hämatologie/Onkologie/Immunologie unter Leitung von Prof. Dr. med. A. Neubauer mit Unterstützung durch Dr. med. A. Burchert und Dr. med. C. Dali ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- und ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Während der Dissertation publizierte Artikel:

Wang Y, Cai D, Brendel C, Barett C, Erben P, et al. Adaptive secretion of the Granulocyte macrophage colony stimulating factor(GM-CSF) protects BCR/ABL-positive progenitors from Imatinib- and Nilotinib-induced apotosis via JAK-2/STAT-5 pathway activation. **Blood**. 2006 Nov 7 (Epub ahead of print)

Cai D**, Wang Y**, Ottmann OG, Barth PJ, Neubauer A, and Burchert A. FLT3-ITD-, but not BCR/ABL-transformed cells require concurrent Akt blockage to undergo apoptosis after histone deacetylase inhibitor treatment. **Blood**. 2006; 107(5):2094-2097.

Burchert A, **Wang Y**, Cai D**,** von Bubnoff N., Paschka P, et al. Compensatory PI3-kinase/Akt/mTor activation regulates imatinib resistance development. **Leukemia** 2005; 19(10):1774-82.

Marburg, den 01.01.2007