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Verfasserin:	Agnes Gstöttenbauer
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Betreuer:	Prof. Dr. Giulio Superti-Furga.

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Summary

The signal transduction and gene expression network of the Bcr-Abl fusion tyrosine kinase, that causes Chronic myelogenous leukemia (CML) are only poorly understood. In this diploma thesis certain aspects of the Bcr-Abl signaling should be analyzed. The project mainly focused on two questions:

One aim was the identification of proteins, that are quickly and prominently up- or down-regulated upon drug induced inhibition of Bcr-Abl to serve as markers for Bcr-Abl activity in Chronic myelogenous leukemia (CML). Such markers could be helpful to easily monitor response to drug therapy of patients suffering from CML. In contrast to current methods, faster adaption of therapy to each individual patient would be possible.

Using microarray analysis, more than 800 genes were identified to be differentially regulated after Dasatinib treatment in K562 cells. Out of this list, 27 genes were validated on RNA level by qRT-PCR including very interesting candidates that have been previously reported to play a role in the development of cancer or to be involved in Bcr-Abl signaling. Two proteins, the surface receptor CD69 and the cytokine IL8 have successfully been studied on protein level. They could be shown to be down-regulated upon Bcr-Abl inhibition by either Dasatinib or Nilotinib, two Bcr-Abl kinase inhibitors approved for treatment of CML patients. CD69 was investigated in K562 cells, down-regulation of IL8 was shown in two different Bcr-Abl expressing cell lines. Similar results could be obtained for serum samples of a small cohort of CML patients, IL8 levels are elevated at the timepoint of diagnosis and decline in the course of drug therapy.

The second part of the project involves the investigation of Bcr-Abl core interactors and their role in Bcr-Abl signal transduction. Detailed knowledge of the Bcr-Abl signaling is important for the development of alternative CML therapy possibilities.

Knock-down experiments of the core interactors should help to reveal their function in signal transduction. RNA interference protocols were successfully established for K562 and UT7 p210 cells. Even though a knock-down was achieved for every single core interactor on protein level, phenotypic changes could not have been observed.

In this thesis, the results are discussed and analyzed in detail, proposals for continuation of the project are given.

Zusammenfassung

Die Signaltransduktion und das Genexpressionsnetzwerk der Bcr-Abl Fusions-Tyrosinkinase, die zu Chronisch Myeloider Leukämie (CML) führt, sind nur im Ansatz aufgeklärt. In der vorliegenden Diplomarbeit wurden bestimmte Aspekte des Bcr-Abl Signalings untersucht. Im Wesentlichen wurden im Rahmen dieses Projektes die folgenden zwei Fragestellungen behandelt:

Zum einen sollten Markergene beziehungsweise Markerproteine für die wirkstoffabhängige Hemmung von Bcr-Abl identifiziert werden. Solche Marker wären hilfreich, um den Therapieerfolg von Patienten, die an CML leiden, zu verfolgen. Im Gegensatz zu derzeitigen Methoden könnte die Therapieart schneller an jeden einzelnen Patienten angepasst werden.

Mithilfe eines Microarrays wurden mehr als 800 Proteine identifiziert, die in K562 Zellen nach Wirkstoffbehandlung reguliert werden. Aus dieser Liste konnten 27 Gene mittels qRT-PCR erfolgreich auf RNA-Ebene verifiziert werden. Einige davon wurden bereits mit verschiedenen Krebsarten in Zusammenhang gebracht oder sind sogar im Bcr-Abl Signaling lokalisiert. Für zwei Proteine, CD69 und IL8, konnte auch auf Proteinebene Regulierung in Abhänigkeit von Behandlung mit Dasatinib oder Nilotinib, zwei zugelassenen Medikamenten für die Behandlung von CML, gezeigt werden. Mittels FACS Analyse wurde bestätigt, dass nach Wirkstoffbehandlung weniger CD69 in K562 Zellen exprimiert wird. Eine Senkung der IL8-Sekretion wurde in zwei verschiedenen Bcr-Abl positiven Zelllinien detektiert. Auch die Untersuchung von Serumsproben einiger CML Patienten zeigte den Trend, dass die IL8-Level bei Diagnose erhöht sind, jedoch im Verlauf der Therapie sinken.

Der zweite Teil des Projekts bestand darin, die Rolle der engsten Bcr-Abl Interaktoren in dessen Signaling zu untersuchen. Für die Entwicklung neuer Therapieansätze ist es wichtig, die Signalkaskaden von Bcr-Abl genau zu kennen.

Knock-down-Experimente der einzelnen Interaktoren sollten helfen, die Notwendigkeit dieser Proteine für die Bcr-Abl Signalübertragung aufzuklären. Protokolle für RNA-Interferenz in K562 Zellen und UT7 p210 Zellen wurden erfolgreich etabliert. Obwohl für alle Interaktoren ein Knock-down auf Proteinebene erzielt werden konnte, wurden keine phänotypischen Auswirkungen festgestellt.

Die Ergebnisse werden in diese Arbeit genau analysiert und diskutiert, Möglichkeiten für die Fortführung beider Projekte werden präsentiert.

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1. Introduction

1.1. Leukemia

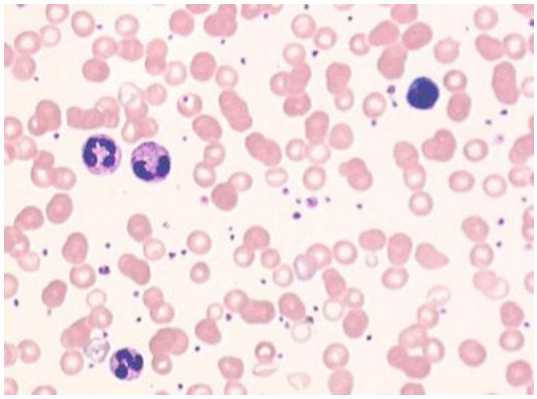
The word “Leukemia” is derived from the greek words “leukos” and “haimos” and means “white blood”. Leukemia describes a form of cancer, that affects hematopoietic stem cells of the bone marrow. The disease is characterized by an increased clonal proliferation of non-mature hematopoietic cells. Several factors including chemicals, ionizing radiation, oncogenic viruses and genetic disposition may increase the risk for disease development [1].

A description of the disease was first given by Rudolf Virchov in 1847. Due to his observations of the unusual composition of the blood with a predominant portion of white blood cells and only a small amount of erythrocytes, he called the disease “Weißes Blut” or “Leukämie”[2].

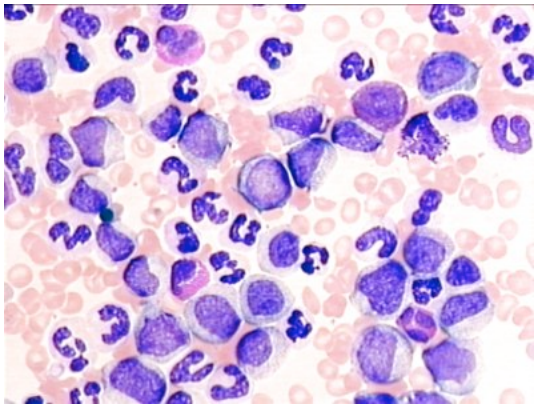
Figure 1.1[1] shows a comparison of normal peripheral blood (a) and the blood of a leukemia patient (b). The blood of the healthy donor contains mainly erythrocytes (red) and only a low number of leukocytes (purple), while the blood sample of the leukemia patient shows high concentration of leukocytes.

The accelerated production of leukocytes leads to an impairment of hematopoiesis as the leukocytes infiltrate the bone marrow. This causes a decrease in erythrocyte and platelet concentration and an imbalance in the composition of blood. As a consequence, patients suffer from anemia, the oxygen transport from the lung to the periphery is insufficient. The lack of platelets impairs blood clotting after injury. The leukocytes are often immature or non-functional and therefore the function of the immune system can also be decreased.

[1] picture from: http://www.deutsches-museum.de/dmznt/ersatzteile/blut/blutbilder/index.html, 30.01.2007



(a) Blood of healthy donor



(b) Blood of CML patient

Figure 1.1: *Accumulation of leukocytes in the blood of a leukemia patient (red: erythrocytes, purple: leukocytes)*

According to disease progression, stage of maturation and lineage of pathologically modified leukocytes, leukemia can be classified into four major types [3]:

- Acute lymphocytic leukemia (ALL)
- Chronic lymphocytic leukemia (CLL)
- Acute myelogenous leukemia (AML)
- Chronic myelogenous leukemia (CML)

Lymphocytic leukemias are characterized by an accelerated production of cells of the lymphocytic lineage (B + T lymphocytes), whereas in myelogenous leukemia, the number of cells of the myeloid lineage (mainly granulocytes) dramatically increases.

Acute leukemias progress very fast and lead to death within a couple of weeks when untreated. They are characterized by an overproduction of immature progenitor cells. The production of healthy mature cells is impaired. Chronic leukemias proceed over a couple of years. Accumulation of mature white blood cells is detected. The disease progresses very slowly and patients suffering from chronic leukemias often need not to be treated in the first stages of the disease [3].

1.1.1. Chronic myelogenous leukemia

Chronic myelogenous leukemia (CML) is a pluripotential stem cell disease characterized by the excessive proliferation of cells of the myeloid lineage. The disease is caused by a reciprocal translocation between chromosomes 9 and 22, leading to the expression of the Bcr-Abl fusion oncogene. The gene product is a constitutively active tyrosine kinase. The Bcr-Abl signaling leads to an accelerated cell proliferation signal.

An overview over the hematopoietic lineage and the development of CML is given in figure 1.2[2]. Hematopoietic stem cells, located in the bone marrow, give rise to the cells of the myeloid lineage (myeloid progenitors) and cells of the lymphoid lineage (lymphoid progenitors). The myeloid progenitors further differentiate into granulocyte/macrophage progenitors or megakaryocyte/erythrocyte progenitors. As the name already implies, granulocyte/macrophage progenitors can either differentiate into mature granulocytes or macrophages while megakaryocyte/erythrocyte progenitors differentiate into erythrocytes or megakaryocytes (which in turn give raise to platelets) [4].

The progression of CML is characterized by three main stages, a chronic stage followed by an accelerated stage ending up in blast crisis. During the chronic phase, with an average duration of 3-4 years the granulocytic cell lineage massively expands. The granulocytes of patients are positive for Bcr-Abl. In blast crisis, cell differentiation is blocked and immature myeloid or lymphoid precursors (also called blast cells) accumulate in the peripheral blood or the bone marrow. Blast crisis leads to death after 3-6 months. Up to now, bone marrow transplantation is the only curative therapy of CML [4, 5].

[2] figure adopted from [4]

The Bcr-Abl protein is necessary for the development and the maintenance of the disease, yet, the switch from chronic phase to accelerated phase and blast crisis requires accumulation of additional abnormalities [4].

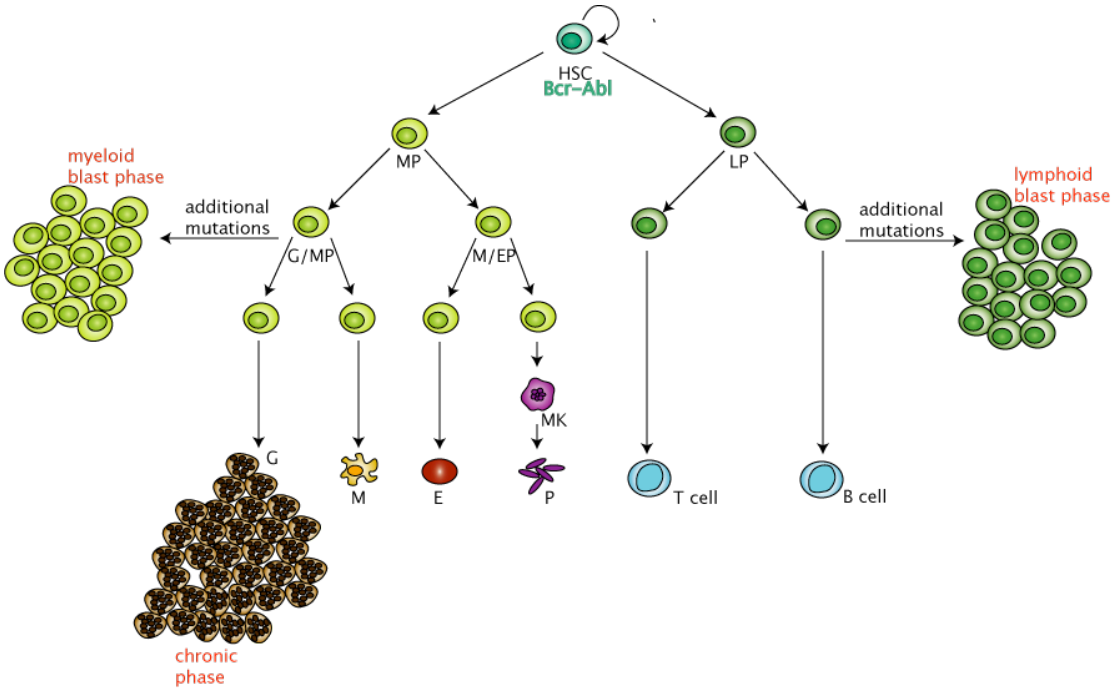


Figure 1.2: *Development of CML (HSC hematopoietic stem cell, MP myeloid progenitor, LP lymphoid progenitor, G/MP granulocyte/macrophage progenitor, M/EP megakaryocyte/erythrocyte progenitor, G granulocyte, M macrophage, E erythrocyte, MK megakaryocyte, P platelet)*

1.1.1.1. Epidemiology

Chronic myelogenous leukemia accounts for about 15% of all adult leukemias. The world-wide case-incidence varies from 1-2 cases per 100 000 persons a year. Incidences increase with age, median age at time of first show is about 40-60. The disease rarely occurs in children. Men are more often diagnosed with the disease, than women (approximate ratio 1,4 : 1) [2, 6].

1.2. The Bcr-Abl fusion protein

CML results from a reciprocal translocation between chromosomes 9 and 22 (t(9;22)(q34;q11)). The translocation leads to the formation of a shortened long arm of chromosome 22 the so-called Philadelphia (Ph) chromosome (see figure 1.3). On the molecular level, this mutation leads to the fusion of the 5′ part the Breakpoint cluster region (Bcr gene) that remains on chromosome 22 to the 3′ part of the c-Abl gene deriving from chromosome 9. The new chimeric gene is called the Bcr-Abl oncogene and it codes for a constitutively active tyrosine kinase [2].

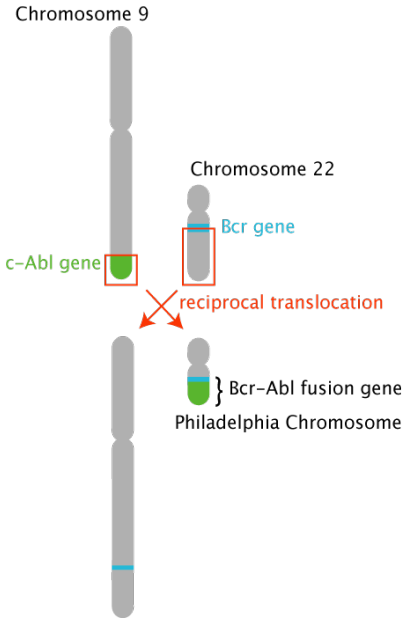


Figure 1.3: *Reciprocal translocation between Chromosomes 9 and 22 leading to the formation of the Philadelphia chromosome carrying the Bcr-Abl fusion gene*

Depending on the exact translocation breakpoints, Bcr-Abl proteins with different molecular weights are formed. Tree main breakpoint regions were identified on chromosome 22. The Bcr-Abl fusion protein is either a 210 kDa-protein (Bcr-Abl p210), a 190 kDa-protein or a 230 kDa-protein. All the forms contain the same part of the c-Abl gene but the Bcr-part varies in size. The vast majority of CML patients express Bcr-Abl p210 [2].

In approximately 20% of all cases of B-ALL (B-cell ALL) the Bcr-Abl protein also appears, called Philadelphia chromosome positive ALL (Ph^+ ALL). Bcr-Abl p190 appears in most of the cases of Ph^+ ALL (about two thirds of cases), Bcr-Abl p210 is detected in one third of the cases [2].

1.2.1. Regulation and signaling of the Bcr-Abl fusion protein

The Bcr-Abl protein is a huge protein carrying a variety of functional domains (see figure 1.4).

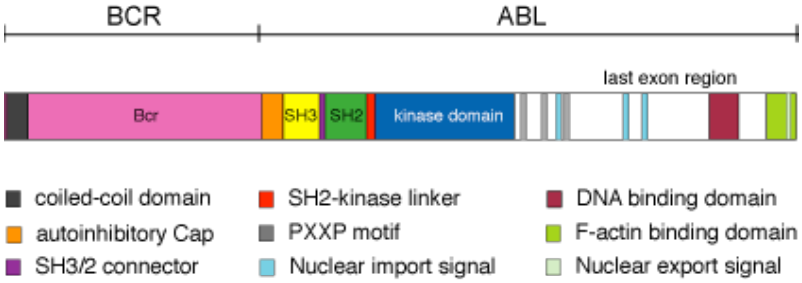


Figure 1.4: *Domains of Bcr-Abl*

1.2.1.1. c-Abl

The c-Abl protein is a non-receptor tyrosine kinase. It has been reported to play a role in regulation of cell growth and survival, oxidative stress, DNA-damage response, actin dynamics and cell migration [7].

c-Abl carries Src-homology-2 (SH2), Src-homology-3 (SH3) and tyrosine kinase domains, which are conserved among members of the Abl family (c-Abl and Arg), Src-family (nine members) and Tec family (five members). The Abl family members furthermore have a long carboxy-terminal tail, the last exon region, which contains protein-protein interaction sites and subcellular localization motifs. Proline rich binding-sites in the tail mediate binding of SH3 domains of adaptor proteins. Furthermore, the last exon region also hosts a DNA-binding domain and an F-actin binding domain. Three nuclear localization signals (NLS) enable nuclear import of c-Abl [7].

The c-Abl kinase is a very tightly regulated protein. The domains interact with each other in a very complex manner, leading to an auto-inhibited form of c-Abl. Amongst others, interactions occur between the SH3 domain and the linker segment, that connects the SH2 domain with the kinase domain. The SH2 domain contacts the kinase domain via hydrogen bonds. In the autoinhibited conformation, the SH3 domain and the SH2 domain form a “clamp” and the activation loop of the kinase domain blocks binding of the substrate and ATP. When

autoinhibited, c-Abl is not phosphorylated on tyrosine residues. In c-Abl 1b, an N-terminal myristoyl group is necessary for maintaining the autoinhibited, non-phosphorylated structure, it remains unclear which moieties substitute the function of the myristoyl group in the alternative spliceform c-Abl 1a that is not myristoylated. Phosphorylation of a tyrosine residue in the activation loop leads to a conformational change and is necessary to activate c-Abl [7].

In the Bcr-Abl fusion protein, the very N-terminal part of Abl is missing, the Bcr-moiety elongates the protein instead. Except from the myristoyl group, all the functional domains of c-Abl are also present in Bcr-Abl and the fusion protein shows very similar intramolecular interactions as c-Abl [7].

1.2.1.2. Bcr

Although the Breakpoint cluster region was excessively studied, the exact function of the Bcr gene remains unclear. Different transcript versions and isoforms have been identified. The protein consists of an N-terminal coiled-coil domain, a Rho-GAP and a Rho-GEF domain. Furthermore, the protein contains binding sites for a number of important signaling proteins [8].

Depending on the translocation breakpoint, part of the Bcr protein can also be found on the Bcr-Abl protein. Through the N-terminal coiled-coil domain, deriving from the Bcr-moiety, Bcr-Abl is able to form dimers and tetramers. This oligomerization enables Bcr-Abl to trans-autophosphorylate the activation loop of Abl, which is the main contribution to its costitutive activity. As for c-Abl this phosphorylation is necessary to activate the kinase [4, 7].

1.2.1.3. Protein-protein interactions and activation of signaling pathways

Bcr-Abl interacts with a large variety of signaling proteins. The fusion of c-Abl and Bcr leads to the formation of a huge platform that permits binding of a large number of proteins that are then phosphorylated and turn on numerous of important signaling pathways (e.g.: Ras-MAPK, PI3K/AKT, Jak-STAT,...), which then activate a range of transcription factors (e.g.: NF-κB, MYC, STATs,...). This changes the gene expression pattern of the cells and leads to hyperproliferation.

The exact mechanism of how these signaling pathways are activated by Bcr-Abl and which proteins are crucial for the signal transduction is only poorly revealed. A number of direct and indirect interactors of Bcr-Abl have been identified, including Grb2, Cbl and subunits of the PI3-kinase [2]. Several core interactors signal into the same pathways. The stoichiometric composition and the exact interaction network of the core interactome remains unclear.

It is still not fully understood, which interactions are crucial to preserve the cancerous signaling of Bcr-Abl.

1.2.2. Inhibition of Abl and Bcr-Abl

1.2.2.1. Imatinib

Imatinib (also called STI571) was the first identified selective inhibitor of the Abl proteins. The phenylaminopyrimidine derivate specifically binds and inhibits autophosphorylation of three kinases: Abl, c-Kit and PDGF-receptor.

Imatinib binds to the ATP pocket when the Abl-kinase domain is in its inactive conformation and the activation loop blocks the ATP binding site. This specific binding is crucial for the selectivity over closely related kinases like Src [9].

The strong inhibitory function of Imatinib on Bcr-Abl induces growth arrest and apoptosis in Bcr-Abl positive cell lines, but not in Bcr-Abl negative cell lines. Mice injected with the human, myeloid Bcr-Abl$^+$ cell line KU812 and treated with Imatinib showed a tumor-free survival, whereas growth of injected U937 cells (a Bcr-Abl negative myeloid human cell line) was not inhibited in growth by Imatinib [10].

During the clinical trails, more than 96% of Imatinib-treated CML patients in chronic phase respond to Imatinib treatment with complete hematologic response (CHR, normalization of white blood cell counts), about 69% of patients showed complete cytogenetic response (CCR, 0% Ph$^+$ cells in bone marrow) after one year treatment [11]. The high inhibitory potency of Imatinib and the relative low side effects led to an exceptionally fast approval of the drug for CML treatment by the US Food and Drug Admimistration (FDA) in 2001, less than three years after start

of Phase I clinical studies. As the drug also inhibits c-Kit, which is an oncogene leading to gastrointestinal stromal tumor (GIST), it was approved for therapy of GIST patients, too. Imatinib was developed by Novartis and is sold under the trade name "Gleevec" [9].

Resistance to Imatinib Availability of Imatinib is a great step in the treatment of CML, still it has some disadvantages. First, constant drug treatment is necessary, as the Bcr-Abl positive clones of hematopoietic stem cells are not completely eliminated. Second, resistances to Imatinib occur. A few patients do not respond to Imatinib treatment at all (primary resistance), others develop resistance after primary response and the disease again progresses (secondary resistance). Mutations in the Bcr-Abl gene kinase domain lead to insensitivity to the drug. These mutations can affect the drug binding residues themselves, as well as distant amino acids leading to conformational changes that prevent Imatinib binding (allosteric inhibition). Both, primary and secondary resistance are more common in patients with an advanced stage of the disease [7, 10].

1.2.2.2. Second generation inhibitors

A lot of effort was made to develop Abl-inhibitors that also target mutated forms of Bcr-Abl. This led to the development of Dasatinib (BMS-354825) and Nilotinib (AMN-107). Both bind, like Imatinib, to the ATP-binding site of the Abl-kinase domain and are able to induce cell death in Bcr-Abl positive cells.

Dasatinib Dasatinib was developed by Bristol-Myers Squibb and it is sold as "Sprycel". Based on another chemical structure than Imatinib, Dasatinib is a less selective kinase inhibitor than Imatinib, also targeting Src family kinases and many other Tyr- and Ser/Thr-kinases. In 2006, Dasatinib was approved for the treatment of CML patients insensitive to Imatinib in the US and in the EU. The drug has a 325-fold greater potency than Imatinib for wild-type Bcr-Abl and leads to hematologic and cytogenetic responses in CML patients in all stages of the disease [5]. Moreover, Dasatinib can inhibit all tested Bcr-Abl resistance mutations except the T315I mutation. Another advantage of Dasatinib is its inhibitory effect on Src family kinases which may play a role in disease progression [12, 13].

Nilotinib Nilotinib, a derivative of Imatinib, developed by Novartis is sold under the trade name "Tasigna". Since 2007 it is approved for the treatment of Imatinib-insensitive CML patients in the US and the EU. Nilotinib is a very selective inhibitor, targeting the same kinases as Imatinib. It binds the Abl-kinase domain more tightly and therefore has 30-50 times greater potency than Imatinib [5]. Like Dasatinib, it is able to inhibit all tested Bcr-Abl mutations except the T315I mutation. Effective Nilotinib therapy was observed during all phases of disease, responses were higher in earlier stages of disease [14].

Other inhibitors Several other potent Bcr-Abl inhibitors have already been developed, currently in pre-clinical or early clinical test phase. Some of them are able to inhibit T315I mutated Bcr-Abl. Another possibility to treat CML is targeting downstream signaling proteins of Bcr-Abl. This requires exact understanding of the Bcr-Abl signaling machinery [5].

2. Aim of the project

2.1. Changes in gene expression induced by Bcr-Abl

As already mentioned, Bcr-Abl is a constitutively active tyrosine kinase, that leads to a permanent pro-proliferative and anti-apoptotic signal. Up to know, the signaling pathways and the target genes of Bcr-Abl are not fully understood. The state of activity and the expression levels of proteins, that regulate the cell cycle must be altered in a way to avoid apoptosis and push growth and cell division. In CML, the differentiation of the myeloid precursors into active, mature cells is impaired by blocking differentiation signaling.

If Bcr-Abl directly alters the activity or expression of a transcription factor, this in turn leads to changes in the expression of the targets of the transcription factor. Certain genes can be either up- or down regulated.

As soon as Bcr-Abl activity is blocked by a specific drug, the signaling is shut down and the cells die after a certain time. The alteration in gene expression in comparison to Bcr-Abl negative cells should come back to a normal level.

2.1.1. Identification of markers for Bcr-Abl activity

The primary aim of the project was to identify a set of biological makers that can be used as a read-out system for drug-dependent Bcr-Abl inhibition in cell lines, as well as in bone marrow or serum from CML patients. The goal was to find a set of proteins that are significantly up- or down-regulated as a consequence of Bcr-Abl inhibition due to drug treatment. Ideally, these proteins should be secreted by the cells or expressed on the cell surface to be easily detectable.

The regulation of the candidate genes should be shown on both, RNA and protein level. A set of potential candidate genes was obtained using a DNA-microarray. First, the data should be confirmed using quantitative real-time PCR (qRT-PCR). As a second step, the regulation should also be shown on protein level using western blotting, flow cytometry analysis or ELISA, depending on the cellular localization of the candidate protein.

Up to now, the effect of drug therapy in CML patients is tested via white blood cell count or analysis of Bcr-Abl expression in leukocytes. The identification of a marker for drug-dependent inhibition of Bcr-Abl after a short period of treatment could help to develop a method to test the effect of the drug faster than via the established methods.

2.1.2. Functional validation of Bcr-Abl core interactors

The signaling of Bcr-Abl runs via signaling cascades involving a broad range of proteins and finally reaches the cell nucleus to change gene expression. The most abundant interactors of Bcr-Abl were previously identified by pull down experiments. The exact binding sites and the stoichiometry of this so-called core interactor complex is not known so far. Another question is, if the interaction between the single core proteins and Bcr-Abl is essential for Bcr-Abl signaling. To approach this problem, the core interactor proteins should be knocked-down using RNA interference. The influence of the knock-down on the survival and proliferation of the cell should give information about the functional involvement in the Bcr-Abl signaling cascade. As the core interactome of Bcr-Abl was identified in K562 cells, the functional validation should also be done in the same cell line.

The determination of critical interactors could lead to the identification of new therapy targets in CML downstream of Bcr-Abl to overcome drug-insensitive Bcr-Abl mutations.

3. Material and Methods

3.1. Cell biology

3.1.1. Cell lines and cell culture

K562	human CML cell line expressing endogenous Bcr-Abl, established from a CML patient in blast crisis
U937	human monocytic cell line, established from a patient with histiocytic lymphoma
UT7 par	human CLL cell line, dependent on GM-CSF
UT7 p210	human CLL cell line, stably transected with Bcr-Abl, independent of GM-CSF

All cell lines were cultured at 37°C in a 5% CO_2 incubator. Cells were kept at subconfluency by splitting them every 2-3 days.

K562 cells, U937 cells and UT7 p210 cells were cultured in RPMI (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Invitrogen). UT7 par cells were supplemented with additional 2 ng/ml human GM-CSF (R&D Systems).

3.1.1.1. RNA interference

For each Bcr-Abl core interactor a SMARTpool siRNA was purchased from Dharmacon. To target Bcr-Abl itself, a b3a2 breakpoint specific siRNA (BAF 7) and a control siRNA with 3 missmatches (BAF 8) were ordered from Dharmacon [15]. According to the manufacturer's instructions the siRNAs were dissolved in siRNA buffer (Dharmacon) to obtain a 20 µM stock solution and kept at -20°C. (See appendix, table A.1 for siRNA sequences of BAF7 and BAF8.)

Gene knock-down in K562 cells The day before transfection confluent cells were split 1:4 to push the cells into logarithmic growth phase. The day of transfection 1x10^6 cells were resuspended in 500 µl medium and seeded into a 6-well plate. 5 µl siRNA solution and 18 µl HiPerFect were combined with 500 µl serum free RPMI. The solution was mixed well by vortexing and incubated for 7 min at room temperature to allow formation of complexes. Then, the siRNA mixture was added to the cells dropwise during shaking of the plate. This leads to an siRNA concentration of 100 nM. 6 hours after transfection 2 ml of medium were added to the cells. Cells were either harvested 48 h after transfection or the transfection procedure was repeated 24 h after primary transfection and cells were finally harvested 72 h after primary transfection.

Gene knock-down in UT7 p210 cells UT7 p210 cells were transfected using the Amaxa electroporation system. 1x10^6 cells were resuspended in 200 µl Amaxa Nucleofactor solution V and 5 µl siRNA were added to the cell suspension (leading to a 500 nM siRNA solution). The mixture was transferred into Amaxa cuvettes and transfected using program X-001 of the Amaxa Nucleofector. Then, 1 ml RPMI + 10% FCS was added to the cell suspension and transferred into a 6-well plate containing 2 ml pre-warmed RPMI +10% FCS and incubated on 37°C. Knock-down was analyzed 48 h after transfection.

3.1.1.2. Drug treatment of cells

The kinase inhibitors Dasatinib (Sprycel, BMS-354825) and Nilotinib (Tasigna, AMN107) were synthesized by WuXi PharmaTech (Shanghai, China). 20 mM stock solutions of the kinase inhibitors were prepared in DMSO and diluted in the respective cell culture medium to final concentrations of 100 nM for Dasatinib and 1 µM for Nilotinib.

Subconfluent cells were treated with the drugs for 3 hours for RNA isolation or up to 24 h for western blot analysis, flow cytometry analysis or ELISA.

3.2. Genomic analysis

3.2.1. RNA extraction from cell lines

RNA was purified using the RNeasy Mini Kit (Qiagen) in combination with QIAshredder columns (Qiagen) following the manufacturer's instructions. If real-time PCR was to be performed, RNA was digested with DNase (Fermentas) to eliminate a potential genomic DNA contamination. To determine RNA quantity, the absorbance was measured at 260 nm in a spectrophotometer. RNA quality was verified by running 5 µl of RNA sample after DNase digestion diluted 1:1,5 in loading buffer on a 1 % agarose (Sigma) gel in TAE and checking for integrity of the 28S and 18S rRNA bands.

Loading buffer	8 ml formamide
	20 µl 0,5 M EDTA
	1,98 ml RNAse-free water
TAE	40 mM Tris
	40 mM acetic acid
	1 mM EDTA

3.2.2. MicroRNA extraction from cell lines

For isolating microRNA (miRNA), the mirVana™ miRNA Isolation Kit from Ambion was used, extraction was performed by following the manufacturer's protocol. This kit allows the isolation of small RNA-containing total RNA, it includes organic extraction followed by purification on a glass fiber filter.

3.2.3. Quantitative real-time PCR (qRT-PCR)

For reverse transcription 1 µg RNA and 0,5 µg oligo(dT)18 primer (Fermentas) in a total volume of 11 µl were heated for 5 min at 70°C, then chilled on ice. Next, 4 µl 5x reaction buffer (Fermentas), 2 µl 10 mM dNTPs, 20 U RNasIn ribonuclease inhibitor (Promega) and water were added to reach a final volume of 19 µl.

The mix was incubated at 37°C for 5 min. 1 µl 200 U/µl RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) was added to each reaction and incubated at 42°C for 1 h. The enzyme was heat-inactivated at 70°C for 10 min.

Quantitative real-time PCR was performed with iTaq SYBR Green Supermix With ROX (Bio-Rad) as detection chemistry using a 7700 cycler (Applied Biosystems) or a Rotor-Gene 6000 (Corbett). Primer sequences are listed in the appendix (table A.2).

For each qRT-PCR reaction 10 µl iTaq SYBR Green Supermix, 0,5 µl 10 µM primer mix, 4,5 µl water and 5 µl 1:20 diluted cDNA were combined by hand or using the CAS-1200 liquid handling system (Corbett). PCRs were run with the following thermoprofile: Initial denaturation at 95°C for 3 min; then 40 cycles of 94°C 30 sec, 60°C 15 sec and 72°C 30 sec. Melting curves were checked after each run to detect potential primer dimers and contaminations. Using GAPDH as a reference gene, evaluation of expression changes was performed via calculation of a standard curve or by applying the 2^-$\Delta\Delta C_T$ method [16].

3.2.4. Quantitative real-time PCR (qRT-PCR) of microRNA 21

To quantify microRNA 21 (miR21) the qRT-PCR-based TaqMan® MicroRNA Assay hsa-miR-21 from Applied Biosystems was performed. This assay includes reverse transcription with a miRNA-specific looped primer to convert the microRNA into cDNA, followed by real-time PCR with cDNA complementary TaqMan® probes. The assay was carried out according to the manufacturer's instructions. RNA isolated with the Ambion RNA extraction kit was used. As a control, the RNU43 assay was used.

3.3. Protein biochemistry

3.3.1. Cell lysates for protein gels

Lysis buffer was prepared by adding the following protease inhibitors to IP-buffer: 50 mM NaF, 1 mM Vanadate, 1 mM PMSF (Sigma), 5 µg/ml TLCK (Roche),

10 µg/ml TPCK (Biomol), 1 µg/ml leupeptin, 1 µg/ml aprotinin and 10 µg/ml soybean trypsin inhibitor (all Roche). Cells were harvested and washed in 10 ml PBS (Invitrogen). Next, the pellet was resuspended in 50 to 200 µl ice cold lysis buffer to reach a concentration of approximately $1x10^5$ cells / µl . The cell pellet was resuspended well to achieve good cell lysis and the mixture was immediately centrifuged for 10 min at 13 000 rpm at 4°C. The supernatant containing the proteins was transferred into a new vial and the protein concentration was measured using Bradford assay (Bio-Rad) according to the manufacturer's instructions using a solution of 1 µg/ml BSA as standard. For normalisation, the samples were diluted in IP-buffer and the appropriate amount of 4 x SDS sample buffer was added to reach a protein concentration of 100 µg/ 20 µl. The samples were heated at 95°C for 4 minutes and directly loaded on the acrylamide gel or stored at -20°C.

IP-buffer	50 mM Tris pH 7,5
	150 mM NaCl
	5 mM EDTA
	5 mM EGTA
	1% NP-40
4 x SDS sample buffer	200 mM Tris- HCl pH 6,8
	40% (v/) glycerol
	8% (w/v) SDS
	brom phenol blue
	1,4 M β- mercaptoethanol

3.3.2. SDS-PAGE

20 µl of cell lysate containing 100 µg total protein were loaded on a discontinuous polyacrylamide gel consisting of 7% - 15% separating gel (depending on size of protein of interest) and 5% stacking gel. As weight marker 5 µl PageRuler Prestained Protein Ladder (Fermentas) were used.

x % separating gel	
30% acrylamide/bisacrylamide	x ml
gel buffer 1	7,5 ml
water	ad 30 ml
10% APS (Sigma)	300 µl
Temed (Sigma)	30 µl
5% stacking gel	
gel buffer 2	10 ml
10% APS (Sigma)	100 µl
Temed (Sigma)	15 µl

The gel was run in running buffer at 60 mA or 90 mA depending on gel size until the running front reached the gel front. The gels were further analyzed by western blotting.

gel buffer 1	1,5 M Tris-HCl pH 8,8 0,4% SDS
gel buffer 2	0,125 M Tris-HCl pH 6,8 0,1 % SDS 5 % acrylamide/ bisacrylamide
running buffer	50 mM Tris-HCl pH 8,5 380 mM glycine 7 mM SDS

3.3.3. Western blots

Acrylamide gels were blotted onto a Protran nitrocellulose membrane (Whatman Schleicher & Schuell) using a semi dry blotting apparatus. The blot was assembled using two filter papers soaked in transfer buffer placed at the anode at the bottom of the apparatus. The papers were followed by the membrane and the gel containing the negatively charged proteins. The gel was covered by two more wet filter papers. To transfer the proteins onto the membrane, the blot was run 1 - 1,5 h at 1 mA/cm^2.

c-Abl and Bcr-Abl blots The nitrocellulose membrane was blocked in blocking solution 1 for 20 min at least. The c-Abl and Bcr-Abl proteins were detected using the mouse antibody specifically against Abl (clone 63-21). The antibody was diluted 1:2000 in blocking solution 1 and incubated for 1 h at room temperature. After washing the membrane 3 x in PBST the primary antibody was detected using an Alexa Fluor 680-labeled goat anti-mouse antibody (Invitrogen) diluted 1:7000 in PBST and incubated 1 h at room temperature in the dark. Finally, the blot was again washed 3 x in PBST. The blot was scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Phospho-tyrosine blots The nitrocellulose membrane was blocked in blocking solution 3 for 20 min at least. The phosphotyrosine residues were detected using the mouse antibody specifically against phosphotyrosine (clone 46-10). The antibody was diluted 1:1000 in blocking solution 3 and incubated for 1 h at room temperature. After washing the membrane 3 x in TBST the primary antibody was detected using an Alexa Fluor 680-labeled goat anti-mouse antibody (Invitrogen) diluted 1:7000 in TBST and incubated 1 h at room temperature in the dark. Finally, the blot was again washed 3 x in TBST. The blot was scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

p57 Kip2 blots The nitrocellulose membrane was blocked in blocking solution 2 for 40 min at least. The p57 Kip2 protein was detected using the rabbit antibody (Upstate, #06-556) diluted 1:500 in blocking solution 2 and incubated at 4°C over night. After washing the membrane 3 x in PBST the primary antibody was detected using an IRDye 800-labeled goat anti-rabbit antibody (Rockland) diluted 1:7000 in PBST and incubated 1 h at room temperature in the dark. Finally, the blot was again washed 3 x in PBST. The blot was scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Grb2 blots The nitrocellulose membrane was blocked in Odyssey blocking buffer (Li-Cor Biosciences) for 40 min at least. The Grb2 protein was detected using a mouse antibody (BD Biosciences #610111) diluted 1:2000 in Odyssey blocking buffer and incubated for 1 h at room temperature. After washing the membrane 3 x in PBST the primary antibody was detected using an Alexa Fluor 680-labeled

goat anti-mouse antibody (Invitrogen) diluted 1:7000 in PBST and incubated 1 h at room temperature in the dark. Finally, the blot was again washed 3 x in PBST. The blot was scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Cbl, Sts1, Ship2 blots The nitrocellulose membrane was blocked in Odyssey blocking buffer for 40 min at least. The proteins were detected using the following primary antibodies: α-Cbl clone 33-77 diluted 1:1000, α-Sts1 clone 34-11 diluted 1:500 and α-Ship2 (provided by Wisniewski[1]) diluted 1:1000 in Odyssey blocking buffer and incubated for 1 h at room temperature. After washing the membrane 3 x in PBST the primary antibody was detected using an IRDye 800-labeled goat anti-rabbit antibody (Rockland) diluted 1:7000 in PBST and incubated 1 h at room temperature in the dark. Finally, the blot was again washed 3 x in PBST. The blot was scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Shc blots The nitrocellulose membrane was blocked in blocking solution 2 for 40 min at least. The Shc protein was detected using a mouse antibody (BD Biosciences, #610878) diluted 1:500 in blocking solution 2 and incubated for 1 h at room temperature. After washing the membrane 3 x in PBST the primary antibody was detected using an Alexa Fluor 680-labeled goat anti-mouse antibody (Invitrogen) diluted 1:7000 in PBST and incubated 1 h at room temperature in the dark. Finally, the blot was again washed 3 x in PBST. The blot was scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

PBST	0,1% Tween-20 (Sigma) in PBS
TBST	0,1% Tween-20 (Sigma) in TBS
blocking solution 1	3% (w/v) BSA in PBST
blocking solution 2	5% blotting Grade Blocker Non-Fat Dry Milk in PBST

[1] Wisniewski et al., Blood 93 (8), 1999

blocking solution 3	5% blotting Grade Blocker Non-Fat Dry Milk in TBST
transfer buffer	2,5 mM Tris 15 mM glycine 10% (v/v) methanol

3.3.4. IL8 ELISA

The Interleukin-8 ELISA kit was bought from BD Biosciences and used according to the manufacturer's instructions. In brief, a 96-well plate was coated with Coating antibody diluted 1:250 in Coating buffer, sealed and incubated over night at 4°C. The next day, the plate was washed 3x with Washing buffer (300 µl/well) and blocked with 200 µl/well Assay diluent. The plate was again sealed and incubated for 1 h at room temperature. After another washing step (3x with Washing buffer, 300 µl/well), 100 µl of each standard, sample and control were pipetted into the appropriate wells. Each standard, sample and control was analyzed in duplicates or triplicates. The standard was prepared using the stock solution and diluted in Assay diluent. If necessary, the samples were diluted between 1:2 and 1:5 in assay diluent as well. The sealed plate was incubated at room temperature for 2 h. Next, the plate was washed again as before, but with 5 washing repeats. Then, 100 µl prepared Working detector were added to each well. Again, the plate was sealed and incubated 1 h at room temperature. The last washing step was repeated 7 times, the wells were soaked in Washing buffer for 30 seconds for each wash. Then 100 µl of Substrate solution (R&D Systems) were pipetted into each well. The plate was incubated up to 30 minutes in the dark without a sealer. The reaction was stopped by adding 50 µl stop solution (2 N H_2SO_4). The absorbance at 450 nm was immediately measured using a microplate reader (Spectra Max M5, Molecular Devices).

Coating buffer	0.1 M Sodium Carbonate pH 9.5 8.4 g $NaHCO_3$ 3.56 g Na_2CO_3
Washing buffer	0.05% Tween-20 (Sigma) in PBS
Assay diluent	10% FCS (Invitrogen) in PBS
Working detector	1:250 Detection Antibody 1:250 SAv-HRP reagent in Assay diluent

3.3.5. Flow cytometry analysis of CD69 surface expression

Cells were harvested and washed in FACS buffer. Approximately $1x10^5$ cells were resuspended in FACS buffer. The PE-labelled mouse monoclonal antibody against human CD69 (#MHCD6904, Caltag/Invitrogen) was added 1:20 to the cell suspension and incubated for 20 minutes on ice in the dark. Then, the cells were washed twice in FACS buffer and resuspended in 30 µl FACS buffer. 5 µl 7'AAD (eBioscience) were added to stain dead cells. The cells were analyzed by flow cytometry using the BD FACSCaliburTM system. 7'AAD negative cells were gated to compare CD69 expression on non-treated and drug-treated cells. Analysis was performed using the BD CellQuest Pro software.

FACS buffer	3% BSA 0.05% NaN_3 in PBS

4. Results

4.1. Marker for drug-dependent Bcr-Abl inhibition

4.1.1. DNA-microarray - candidate identification

The first approach for finding molecular markers for Bcr-Abl activity was a microarray experiment. The expression pattern of K562 cells grown under normal conditions was compared to the expression pattern of K562 cells, that were treated with 100 nM Dasatinib for 3 h. For this experiment the GeneChip ® Human Genome U133 Plus 2.0 Array was used. The statistical analysis of the obtained data set revealed, that more than 1600 genes could be determined as differentially expressed after drug treatment compared to the non-treated control cells. The majority was down-regulated and only 18% of all regulated genes were up-regulated after the treatment. The divergence ranged from 0.01-fold change (most prominently down-regulated gene upon drug treatment) to 5.51-fold change (most prominently up-regulated gene).

From this large dataset a selection of genes was chosen to be further investigated. Selection of candidates was performed under the aspects of degree of expression change and properties of the protein. Preferentially, the protein should be either localized on the cell surface or secreted by the cell to be fast and easily detectable. Finally, a list of 33 genes was generated, 26 of them down-regulated after drug treatment, 7 up-regulated. Figure 4.1 shows a scheme of a cell, illustrating the cellular localization of all the candidates and their relation to the Bcr-Abl machinery as far as this is known [2, 17].

The candidate proteins show very diverse properties concerning cellular localization and function. The list contains secreted and membrane spanning proteins

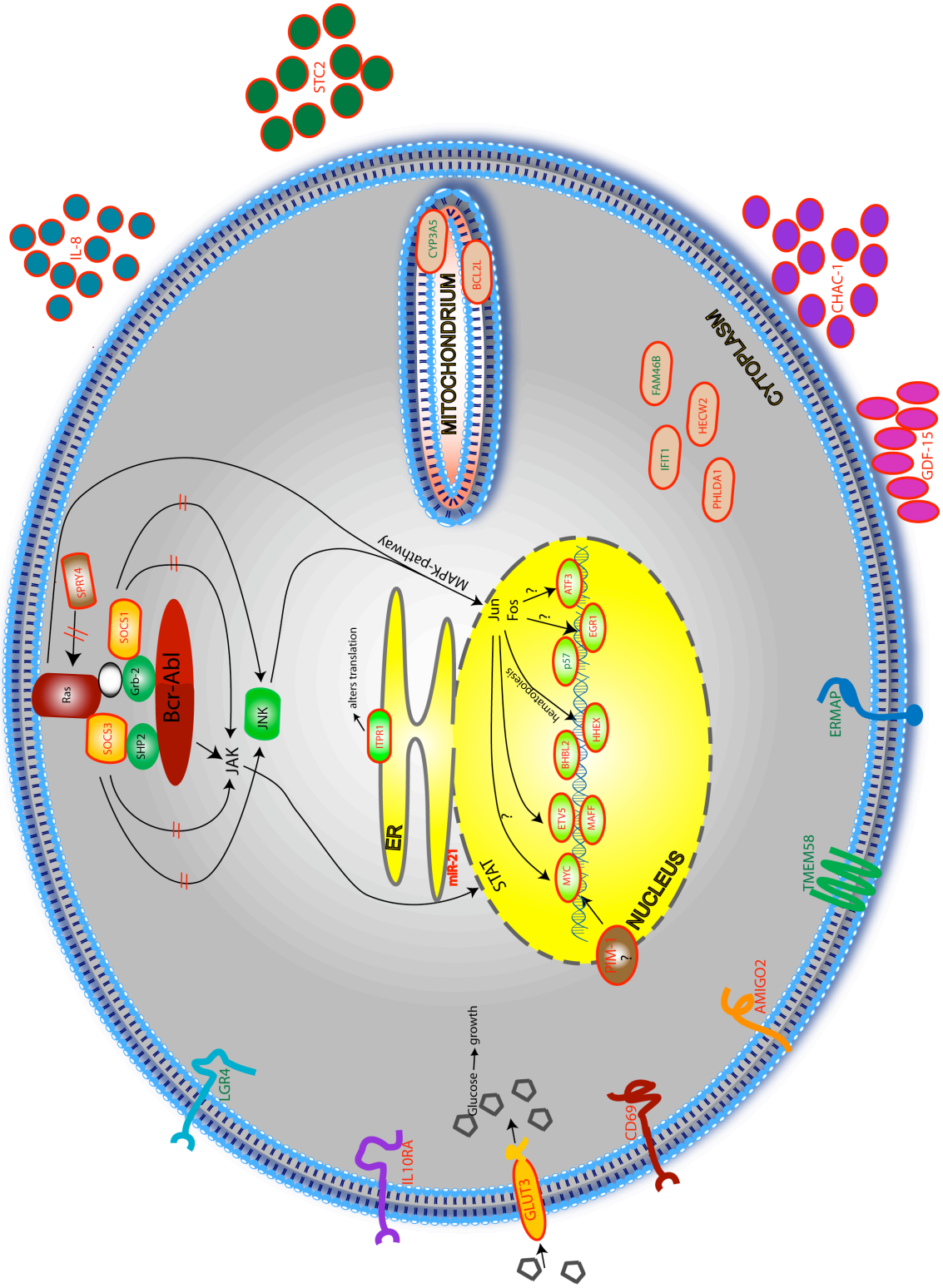


Figure 4.1: *Scheme of relation of marker candidates to Bcr-Abl*

as well as cytoplasmic or nuclear located proteins and proteins embedded in the membrane of the endoplasmatic reticulum or mitochondria. Some of the candidates can be directly linked to Bcr-Abl. They are known to bind and eventually influence Bcr-Abl itself or its binding partners (e.g. SOCS1, SOCS3, SPRY4). For another subset of candidates no direct link to Bcr-Abl is known (e.g. IL10RA, CD69, IFIT1). The list also contains proteins that are very poorly described at all (e.g. TMEM 58, ERMAP, AMIGO2) [17].

Hakansson et al. [18] transfected the Bcr-Abl oncogene to U937 cells and compared the expression pattern of parental and Bcr-Abl transfected cells by microarray analysis. They identified 61 up-regulated genes in the Bcr-Abl positive U937 cells. Interestingly, the list of the proteins they identified as up-regulated after Bcr-Abl induction partly overlaps with our list of down-regulated genes after Bcr-Abl inhibition (e.g. CISH and PIM1). This provides strong evidence, that the oncogene itself influences the expression regulation machinery of these proteins. Paradoxically, they reported IFIT1 to be up-regulated in Bcr-Abl transfected U937 cells, we found IFIT1 to be up-regulated K562 cells after drug-dependent Bcr-Abl inhibition.

4.1.2. Confirmation of the microarray results on RNA level using quantitative real-time PCR

To confirm the microarray data on RNA level quantitative real-time PCR (qRT-PCR) was performed. The investigation was extended by not only comparing non-treated K562 cells to Dasatinib-treated cells, but also to Nilotinib-treated cells. Dasatinib was again used at a concentration of 100 nM as for the microarray experiments, Nilotinib was used at a concentration of 1 μM . At these concentrations, tyrosine phosphorylation could no longer be detected in K562 cells by phosphotyrosine specific western blotting.

Figure 4.2 shows the comparison of the results from the microarray experiments and the qRT-PCR experiments (see appendix, table A.4 for exact values).

Changes in the expression pattern of K562 cells upon treatment with Bcr-Abl inhibitor drugs were investigated on RNA level via two different methods, microarray and qRT-PCR. For most of the candidates, the expression changes from

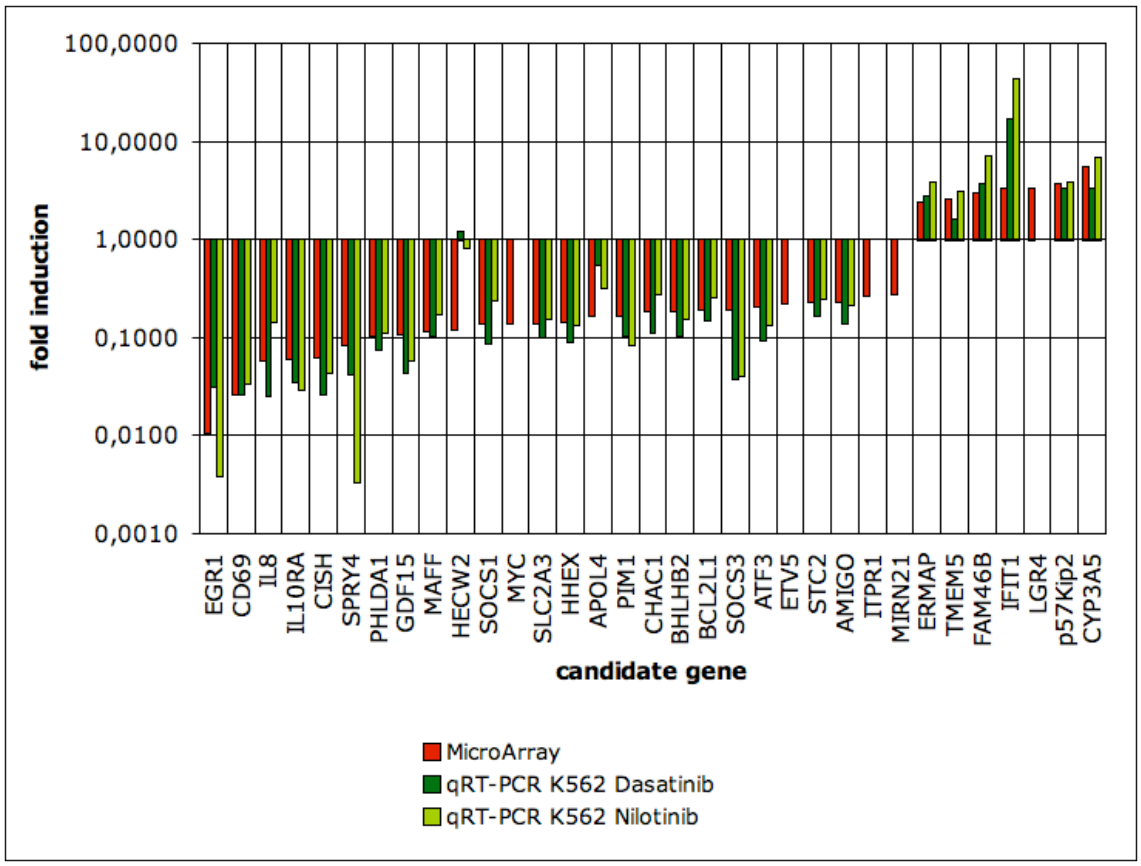


Figure 4.2: *Comparison of microarray results and qRT-PCR results for drug dependent regulation of candidate expression*

both methods match very well (CD69, IL10RA, CISH, PHLDA1, GDF15, MAFF, SOCS1, SLC2A3, HHEX, BHLHB2, BCL2L1, ATF3, STC2, AMIGO2, PIM1, ERMAP, FAM46B, p57Kip2, CYP3A5). For some others (EGR1, IL8, SPRY4, APOL4, CHAC1, SOCS3, TEMEM56, IFIT1) the results from the qRT-PCR did not correlate that well with those of the microarray. However, the tendency of regulation could be confirmed for nearly all the candidate genes. HEC2W, MYC, ETV5, ITPR1 and LGR4 could not be successfully analyzed by qRT-PCR.

Treatment with Dasatinib and Nilotinib led to very similar regulation effects for all the candidates. Although Dasatinib has a broad target spectrum and inhibits a number of important kinases and Nilotinib is a very specific inhibitor for Bcr-Abl, both drugs have the same influence on the expression of candidate genes. As there is very little overlap in the targets of Dasatinib and Nilotinib [19], these results strongly indicate, that the changes in the expression levels of the candidate genes are very likely to be regulated via Bcr-Abl and not via some other targets

of the drugs.

To assure, that the expression change of the candidates is an effect of specific Bcr-Abl inhibition by both drugs, we performed the same qRT-PCR experiments using U937 as a control cell line (only for a subset of candidates). U937 cells do not express the Bcr-Abl fusion gene. Therefore the drug treatment should not affect the expression of the candidate genes. U937 cells were cultivated at the same concentrations of Dasatinib or Nilotinib for 3 h. Again, the expression level of the candidate genes in non-treated U937 cells was compared to the levels in treated cells (see figure 4.3, a table showing the exact data is provided in the appendix, table A.4).

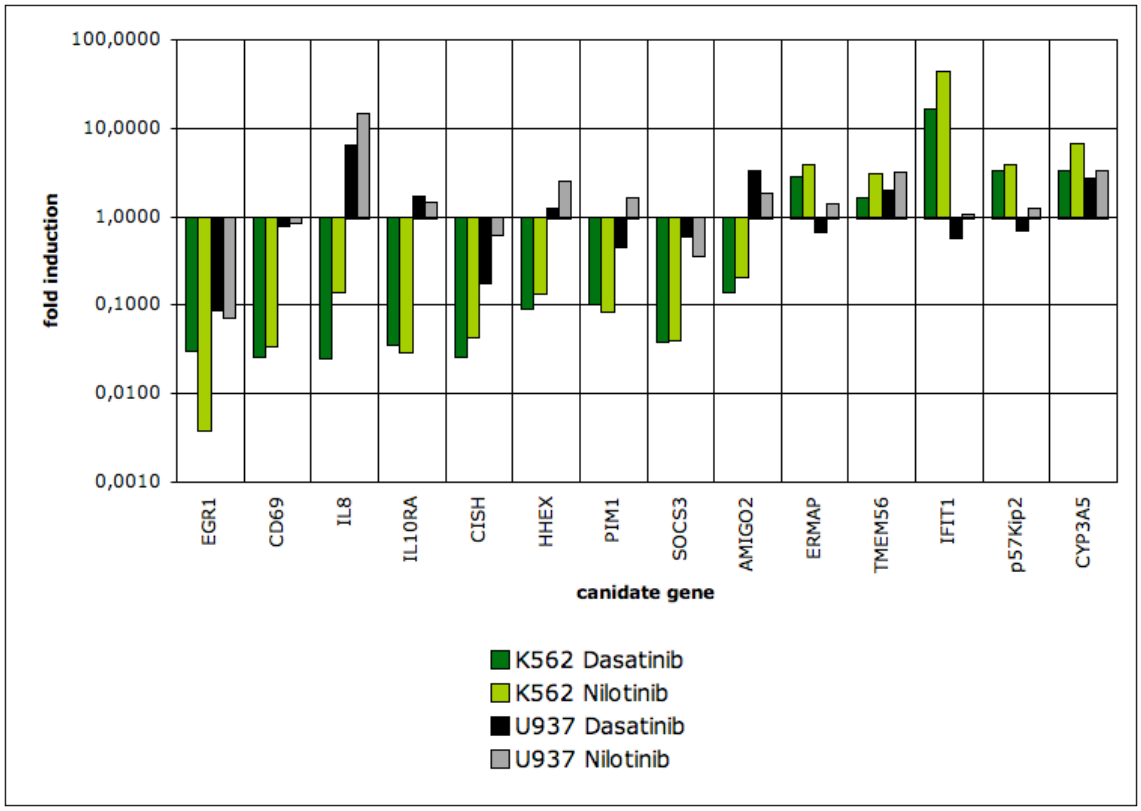


Figure 4.3: *qRT-PCR results of U937 cells vs. K562 cells*

Drug treatment has only a small effect on the expression level of the majority of tested candidate genes in U937 cells. These results again provide strong evidence, that the regulation of most of the candidate genes is a consequence of Bcr-Abl inhibition. Significantly high changes were obtained IL8. For this candidates, drug treatment has an opposite effect in U937 than in K562 cells. Prominent changes in the expression after drug treatment of U937 cells could also be detected for EGR1

and CISH. However, the effect is much higher in K562 cells. The candidate genes TMEM and CYP3A5 are regulated in the same range in K562 cells as well as in U937 cells. They must be suspected to be regulated via another target protein of Dasatinib and Nilotinib and are therefore excluded from the list of possible markers for drug-dependent inhibition of Bcr-Abl.

4.1.2.1. MicroRNA 21

MicroRNA 21 (miR21) was also detected to be down-regulated upon drug treatment in the microarray experiment (0,28-fold induction). For investigation of miRNAs with qRT-PCR special methods need to be used for RNA preparation and reverse transcription. Using the Ambion miRVana miRNA Isolation Kit and the TaqMan MicroRNA real-time PCR Assay from Applied Biosystems, down-regulation of miR21 (0,55-fold change) could be detected upon drug treatment in K562 cells whereas the treatment causes slight up-regulation in U937 cells (1,3-fold change) (see figure 4.4).

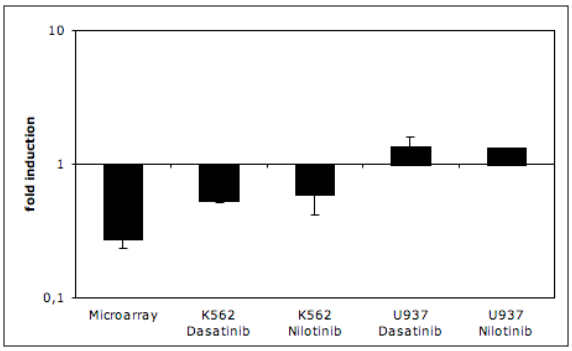


Figure 4.4: *miR21 expression in K562 cells and U937 cells*

MicroRNA21 expression seems to be influenced by Bcr-Abl activity. However, miRNA detection is expensive and time-consuming, therefore miR21 can not be used as diagnostic marker.

4.1.3. Bcr-Abl knock-down in K562 cell line

If the Bcr-Abl fusion protein is responsible for the regulation of the candidate genes, knock-down of Bcr-Abl should have the same influence on the expression of the candidates as inhibition of Bcr-Abl by drugs.

A protocol for transient siRNA transfection of K562 cells was successfully established in the laboratory. Bcr-Abl knock-down in K562 cells was achieved using an siRNA homologous to the breakpoint region of the Bcr-Abl p210 gene (BAF 7). This siRNA leads to a knock-down of Bcr-Abl but it does not target c-Abl or Bcr. As a control, a missmatch siRNA with 3 altered bases was used (BAF 8) [15].

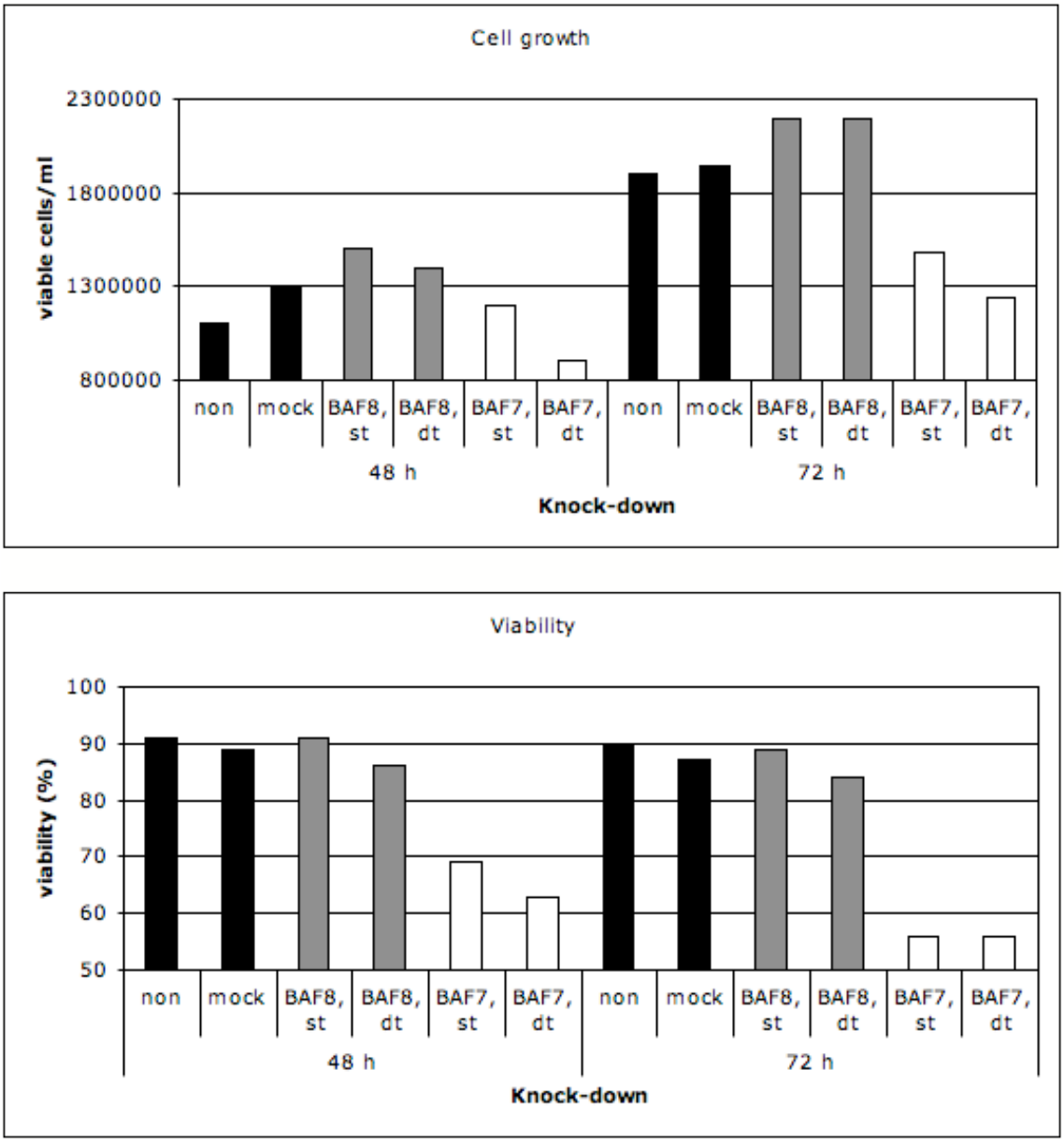
A knock-down of Bcr-Abl to 30% expression could be achieved (not shown). In contrast to the cells that were transfected with the control siRNA, the Bcr-Abl knock-down cells stopped proliferating and died after 48 h (see figure 4.5).

It was not possible to rescue the cells by providing a growth factor cytomix containing Interleukin-3 (IL-3), Interleukin-6 (IL-6), Stem cell factor (SCF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), each of them at a concentration of 1 ng/ml. Growth and viability of Bcr-Abl knock-down cells did not recover when cultivating the cells in cytomix. The cells do not respond to the growth factors. It is possible, that K562 cells do not express sufficient receptors for these growth factors as the survival signal of Bcr-Abl is normally sufficient for the cells to grow. The K562 cells seem to be completely oncogene addicted. The cells are dependent on the growth and survival signals induced by Bcr-Abl.

4.1.4. Investigation of candidate p57 Kip2 on protein level

On RNA level the potent tumor supressor gene p57 Kip2 was up-regulated upon drug treatment. As long as the Bcr-Abl oncogene is active, this negative regulator of proliferation is kept at low level. The microarray data as well as the qRT-PCR results for Dasatinib and Nilotinib treatment showed a 4-fold up-regulation of the mRNA after 3 hour drug treatment. For the investigation on protein level, K562 cells were treated with the drugs for 3 to 24 hours. As shown in figure 4.6, western blot analysis did not show up-regulation of the protein on any time point after drug treatment.

Although the mRNA level has increased to the 4-fold after 3 hours of drug treatment, the amount of the protein remains unchanged. It is not possible to examine p57 Kip2 levels after extended drug treatment as K562 cells die due to the inhibition of the oncogene. p57 Kip2 can not be used as marker for drug-dependent Bcr-Abl inhibition on protein level.



(a)

Figure 4.5: *Growth and viability of Bcr-Abl knock down cells*
BAF8: missmatch siRNA, BAF7: siBcr-Abl, st: single transfection, dt: double transfection (second transfection after 24 h)

4.1.5. Investigation of candidate CD69 on protein level

The second candidate investigated on protein level was CD69. As a surface receptor protein, CD69 would be a suitable marker for Bcr-Abl activity as it can easily be detected by flow cytometry.

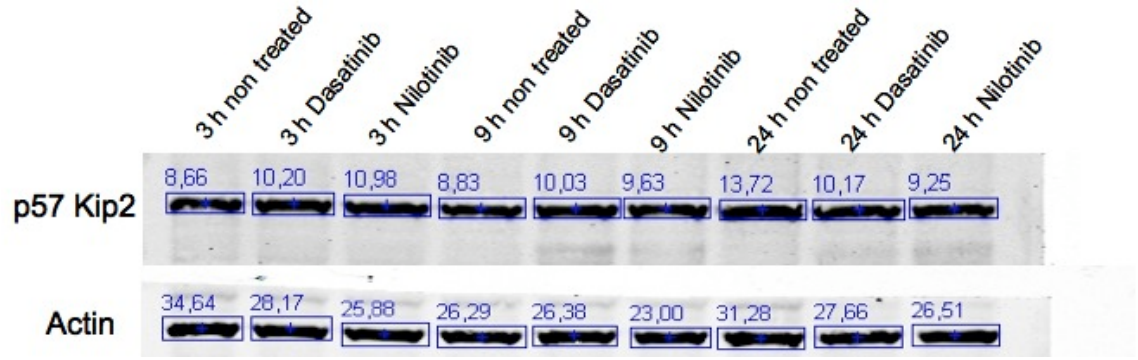
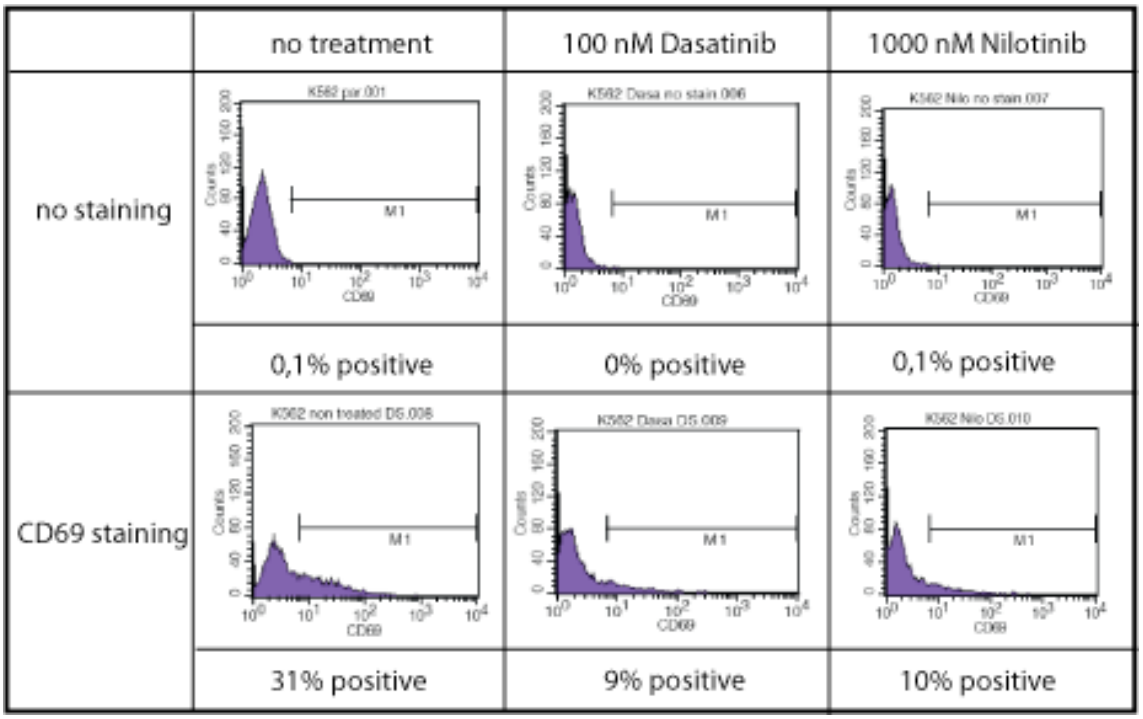


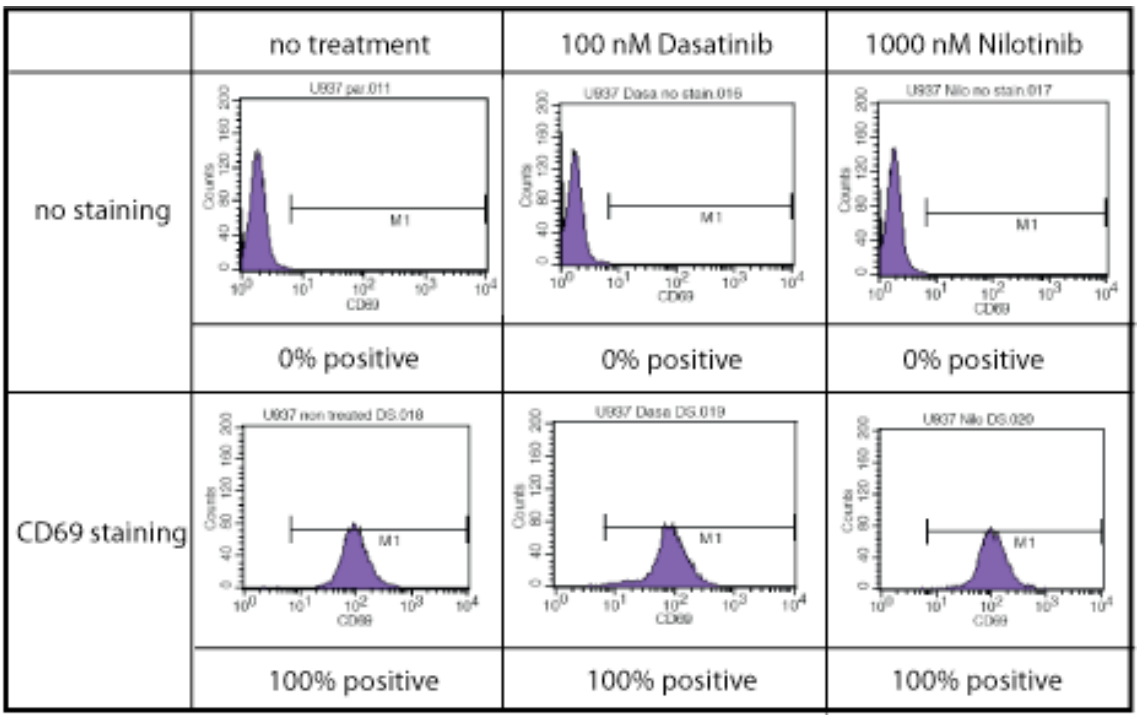
Figure 4.6: *Quantification of p57 Kip2 in K562 cells after drug treatment by western blotting*

On RNA level, CD69 was down-regulated to around 3% expression (= 40-fold down-regulation) upon drug treatment of K562 cells, whereas no significant effect could be detected in U937 cells. Expression on protein level was investigated by flow cytometry analysis after 16 h of drug treatment. The cells were stained for CD69 and treated with 7'AAD to distinguish between living and dead cells. CD69 expression was examined on K562 cells as well as on U937 cells as a control. Figure 4.7(a) shows, that down-regulation of CD69 in K562 cells upon drug treatment could be detected by flow cytometry analysis. A reduction from 30% to 10% CD69-positive K562 cells could be observed. The treatment did not affect CD69 expression on U937 cells. Independently of drug treatment, U937 cells always stay 100% positive for CD69 (see figure 4.7(b)). The fact, that the CD69 signal is lower in untreated K562 cells than in untreated U937 cells may be cell type specific, K562 cells may generally express less CD69 protein on their surface.

The autofluorescence of non-stained K562 cells changes upon drug-treatment, the peak of Dasatinib- and Nilotinib-treated, non-stained cells is slightly shifted to the left in comparison to non-treated, non-stained cells (fig. 4.7(a)). Inhibition of Bcr-Abl and the subsequent changes in expression pattern and composition of proteins may be sufficient to alter the autofluorescence of the cell. Similar changes in autofluorescence can not be observed in U937 cells, the drugs affect the cells to a smaller extent (fig. 4.7(b)).



(a) CD 69 in K562 cells



(b) CD 69 in U937 cells

Figure 4.7: *CD69 expression in K562 cells and U937 cells after 16 h drug treatment*

4.1.6. Investigation of candidate Interleukin-8 on protein level

Interleukin-8 (IL8) is a secreted cytokine and plays an important role in mediating inflammatory response. IL8 would be an appropriate marker for drug response as it is easily and rapidly detectable in supernatants as well as in blood serum by ELISA.

4.1.6.1. Interleukin-8 expression in K562 cells

For the analysis of the Bcr-Abl dependent IL8 expression, K562 cells and U937 cells were treated with either Dasatinib or Nilotinib for 3 h to 24 h and the supernatants were analysed by ELISA.

The results show, that non-treated K562 cells express about 4,5 times more IL8 than non-treated U937 cells. This can be due to Bcr-Abl expression, that is not present in the U937 control. K562 cells react to Dasatinib and Nilotinib treatment with reduction of IL8 secretion. Figure 4.8 shows the IL8 levels in the supernatants normalized to 1 x10^6 cells/ml at timepoint of harvest (data see appendix, table A.6).

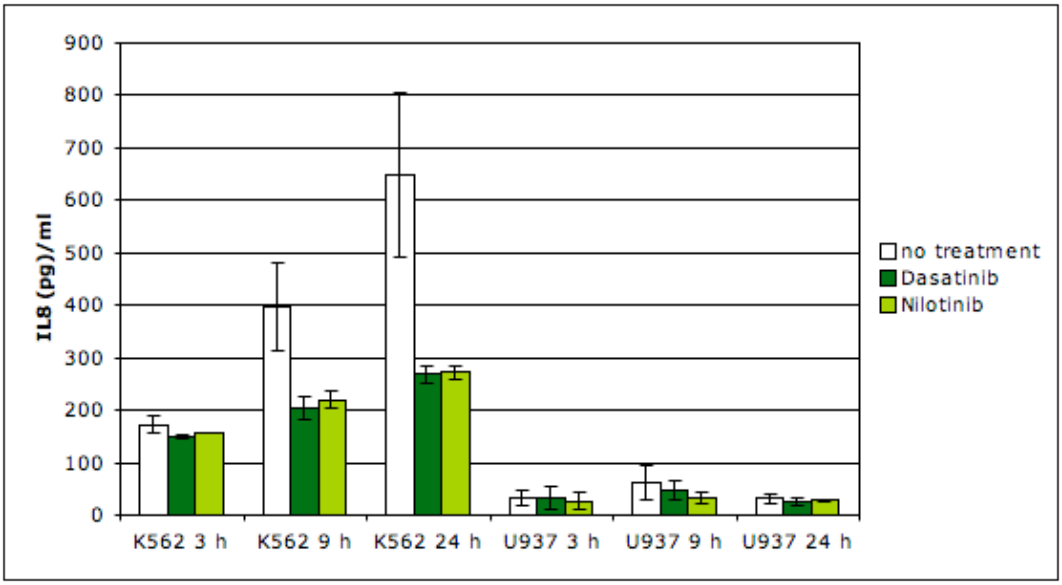


Figure 4.8: *IL8 secretion upon drug treatment in K562 and U937 cells*

After 3 h of drug treatment, the IL8 levels are still the same in non-treated and treated K562 cells. After 9 h treatment, the IL8 levels are reduced to half amount

in treated K562 cells. After 24 h this difference is even more prominent. Drug treatment does hardly affect the U937 cells, the IL8 levels remain the same for treated and non-treated cells at every time point.

4.1.6.2. Interleukin-8 expression in UT7 cell line

Dasatinib sensitivity of UT7 p210 UT7 cell line (UT7 par) is a GM-CSF dependent cell line. Stable transfection of UT7 cells with the Bcr-Abl oncogene (UT7 p210) renders them GM-CSF independent. The Bcr-Abl oncogene induces a proliferation signal in the UT7 cells, which replaces the signal of GM-CSF. Growth of UT7 par (in medium containing GM-CSF) is not affected by Dasatinib, whereas UT7 p210 growing under GM-CSF starvation die at a concentration of 100 nM Dasatinib (see figure 4.9). It was not possible to rescue Dasatinib treated UT7 p210 cells by providing GM-CSF (not shown).

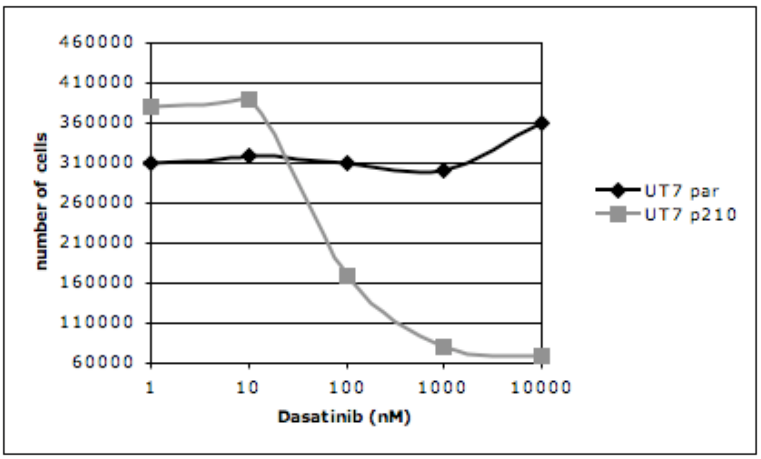


Figure 4.9: *Growth of UT7 par and UT7 p210 cells in increasing concentration of Dasatinib*

Regulation of IL8 secretion upon drug treatment UT7 par and UT7 p210 cells were treated with the same concentrations of Dasatinib (100 nM) and Nilotinib (1 µM) as K562 and U937 cells and the IL8 levels in supernatant were again analyzed by ELISA. In the UT7 p210 cells the IL8 secretion level is significantly higher compared to UT7 par. Figure 4.10 shows, that UT7 p210 cells secrete 3 times more IL8 than the parental cells. Drug treatment does not influence IL8 expression in UT7 par cells, whereas in UT7 p210 cells IL8 levels are reduced to 30% after 3 h

of drug treatment. The effect of drug treatment is even more prominent after 16 h and 24 h. Bcr-Abl inhibition in UT7 p210 cells leads to down-regulation of IL8 secretion to similar amounts as the parental cell line UT7 par (exact data values provided in appendix, table A.7).

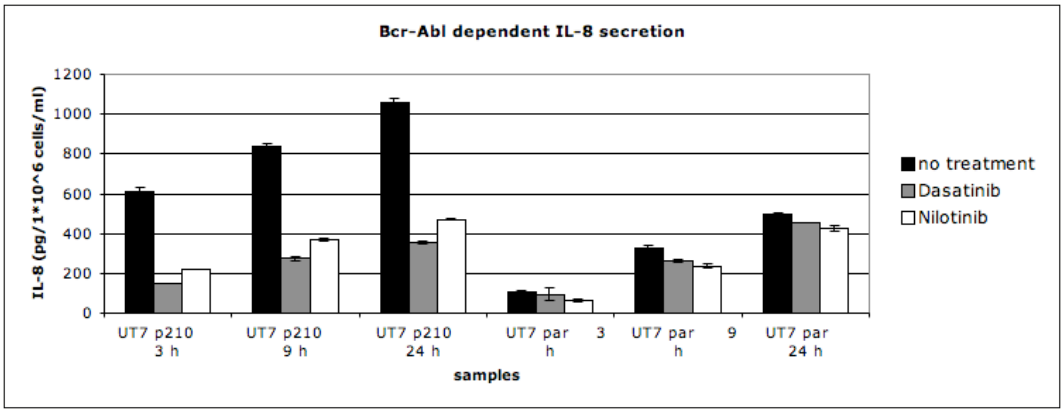


Figure 4.10: *IL8 secretion upon drug treatment of UT7 par and UT7 p210 cells*

4.1.6.3. IL8 in CML

Bcr-Abl dependent expression of IL8 was also tested in blood serum from 4 CML patients. In preliminary experiments, the IL8 expression in serum from newly diagnosed CML patients was compared to their serum IL8 levels during drug therapy. The CML patients, whose samples were used for the study were on Imatinib therapy, as this is the first line therapy for the disease. Dasatinib treated patients are very rare, Nilotinib treatment is only permitted since November 2007 in Austria. As controls, serum samples from 2 healthy donors were also tested. Results are shown in figure 4.11 (see appendix, table A.8 for exact data values).

Around 10 pg/ml of IL8 could be detected in serum samples of healthy donors. This value was taken as a threshold for analysis of IL8 levels in patient samples.

All CML patients showed elevated IL8 levels at time of diagnosis in comparison to the healthy donors. IL8 levels CML patients 1 and 4 normalize already 1 month after Imatinib therapy and stay down after 3 months. Patient 2 shows normalization of IL8 after 3 months, but the levels are again up-regulated after 12 months therapy. This patient does not show complete cytogenetic response even after 12 months Imatinib therapy. Serum IL8 levels of patient 3 also normalize after

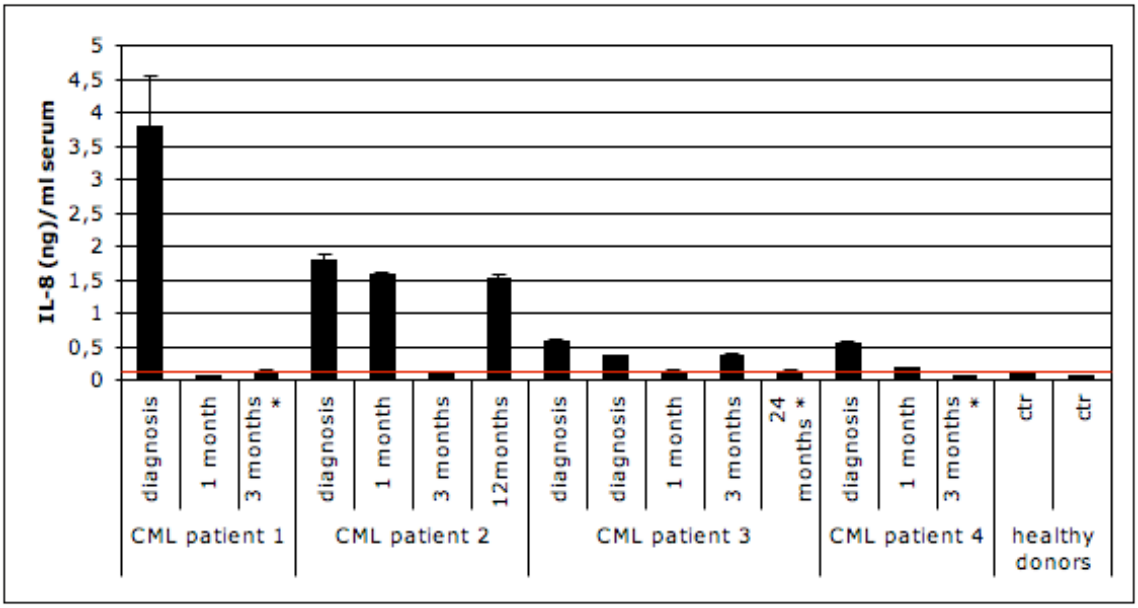


Figure 4.11: *IL8 levels in serum from CML patients (* compete cytogenetic response)*

already 1 month. After 3 moths of therapy, IL8 secretion is again increased, but re-normalized after 24 months when complete cytogenetic response was reached.

These results support the therory, that Bcr-Abl leads to an increase of IL8 in the serum of CML patients. Still, these are preliminary data and the IL8 levels were not normalized to the white blood cell counts.

4.2. Role of Bcr-Abl core interactors in Bcr-Abl signaling

Bcr-Abl is a very potent oncogene, it is sufficient to induce cancerous survival, growth and proliferation signals in cells. Bcr-Abl closely binds and interacts with a variety of proteins that are known to be closely linked to important signaling pathways in the cell.

Several direct and indirect interaction partners of Bcr-Abl have already been identified by large-scale IP. Tandem affinity purification (TAP) using TAP-tagged Bcr-Abl as bait and subsequent mass spectrometry analysis of the purified proteins was performed previously in the lab. The list of proteins identified overlaps

very well with known interactors, some unknown binding partners could also be found. According to the pulldown experiment, the most abundant interactors of Bcr-Abl were chosen to be examined in particular (Brehme, M., Hantschel, O., not published):

- Grb2
- CrkII
- Cbl
- Shc
- Ship2
- Sts1
- p85 α
- p85 β

It is not clear until now, which interactions are crucial for the Bcr-Abl signaling. By knocking down each of these core interactor proteins critical signal mediators of Bcr-Abl should be identified.

4.2.1. Knock-down of Bcr-Abl core interactors in K562 cells

Knock-downs were achieved by transfecting K562 cells with Dharmacon SMART-pool siRNAs using HiPerFect transfection reagent. Each core interactor protein could successfully be targeted and its expression could be reduced to 20% at least (see figure 4.12 and table 4.1).

Growth and viability of K562 cells were monitored using the CASY Counter system. Table 4.1 shows the knock-down efficiencies of the target proteins and the viability of the cells. It was not possible to detect a growth reduction after core interactor knock-down. Cells died after knock-down of Bcr-Abl and c-Abl.

The knock-down samples were also tested for tyrosine phosphorylation by western blot analysis using a specific anti-phosphotyrosine antibody. Figure 4.13 shows, that tyrosine phosphorylation was not affected by any knock-down. The tyrosine phosphorylation of the cell, which is fully induced by Bcr-Abl, is not significantly changed upon knock-down of any Bcr-Abl core interactor.

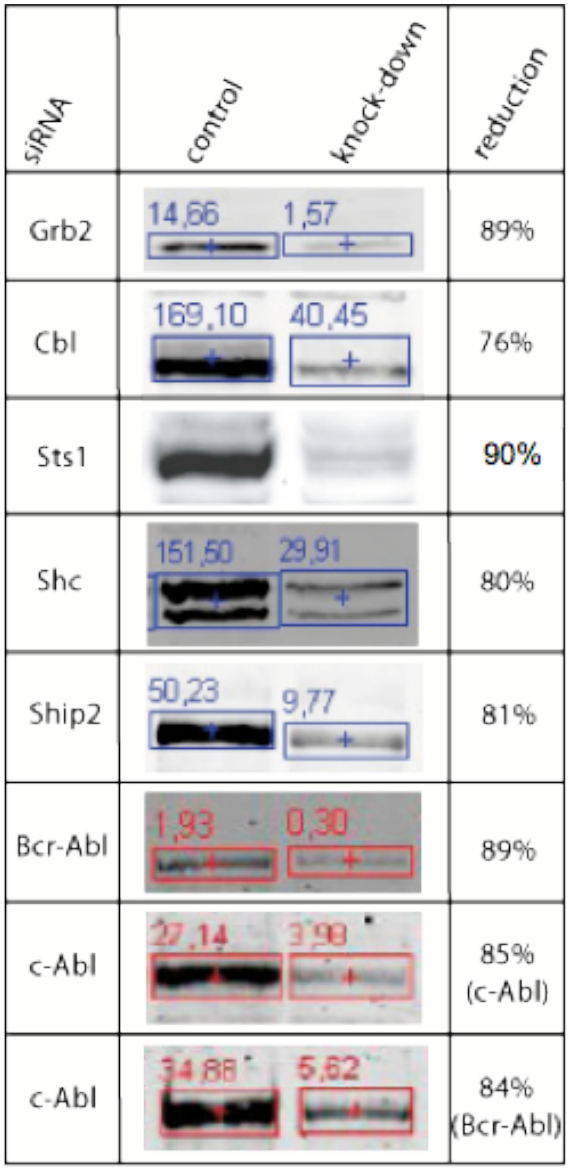


Figure 4.12: *Knock-down efficiency of core interactors in K562 cells determined by western blotting*

The missing band in the lane where the lysate of the Ship2 knock-down was applied is Ship2 itself, the signal is lost because of elimination of the protein. The upcoming band in lane 3, where the control knock-down was loaded and in the last lane could not be identified.

Table 4.1: *Knock-down efficiency of core interactors in K562 cells determined by qRT-PCR and western blotting and viability of knock-down cells*

core interactor	Knock-down efficiency determined by qRT-PCR (%)	Knock-down efficiency determined by western blot (%)	viability (%)
Bcr-Abl		89	60
c-Abl		85	60
Grb2	87	89	90
CrkII	80		90
Cbl		76	90
Sts1		75	90
Shc	90	80	90
Ship2		81	90
p85α	85		90
p85β	97		90

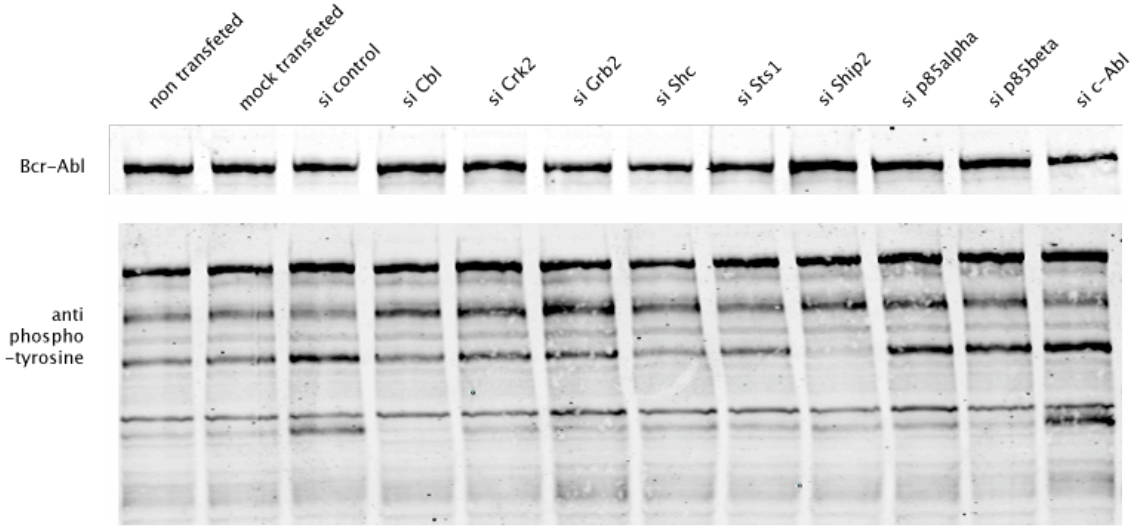


Figure 4.13: *α-phospho-tyrosine blot of K562 knock-down cells*

4.2.2. Knock-down of Bcr-Abl core interactors in UT7 p210 cells

Knock-down of core interactors was also performed in UT7 p210 cells. Transfection of Dharmacon SMARTpool siRNA into UT7 p210 cells using the Amaxa electroporation system was sucessfully estblished. Figure 4.14 shows the knock-

down efficiencies for all proteins where knock-down was performed. Growth of the cells was only monitored by eye, an effect of a knock-down could not be observed.

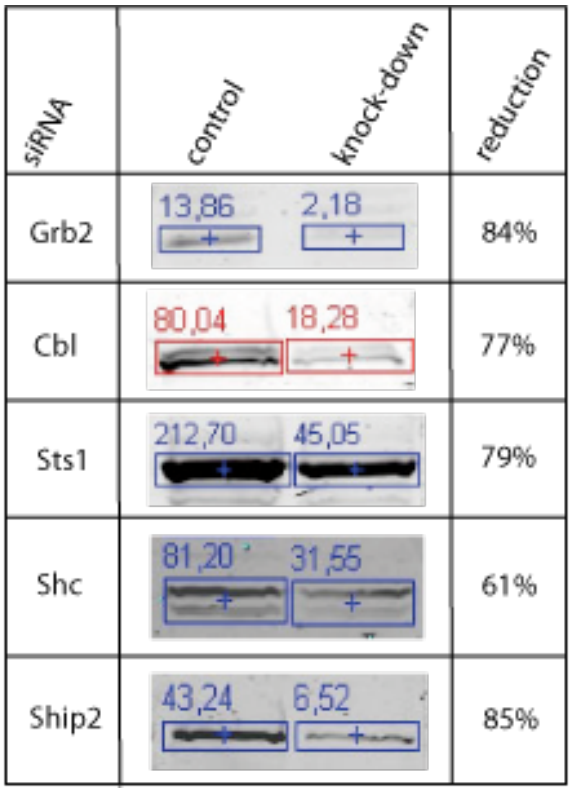


Figure 4.14: *Knock-down efficiency determined by western blotting*

5. Discussion

5.1. Identification of markers for Bcr-Abl activity

The constitutively active tyrosine kinase Bcr-Abl tightly interacts with a variety of proteins and activates a broad range of important cellular signaling pathways. The transcription factors at the end of these signaling cascades influence the regulation of numerous genes. The Bcr-Abl signaling not only regulates the expression of genes required for the survival and growth signaling, it also leads to prominent up- or down-regulation of other target proteins that do not necessarily contribute to the cancerous properties of Bcr-Abl positive cells. When Bcr-Abl is inhibited by treatment of the cells with specific drugs, the levels of Bcr-Abl dependent genes should come back to a normal expression level.

Up to know, the effect of drug therapy in patients can only be determined via white blood cell count or examination of Bcr-Abl positive cell clones in serum or bone marrow by FISH or cytogenetic analysis using RT-PCR. It takes around one month until the effects of therapy are detectable. For CML patients and doctors it would be very convenient if the duration until the result of therapy can be determined was shortened. This would allow a faster adjustment of therapy for every single patient.

5.1.1. Candidate investigation on RNA level

Using a microarray, more than 1600 genes turned out to be differentially regulated in K562 cells with inhibited Bcr-Abl compared to cells with an active oncogene. For 27 of these candidates a Bcr-Abl dependent regulation could be confirmed on RNA level by qRT-PCR. Two proteins, Interleukin-8 and CD69, could be shown to be Bcr-Abl dependently regulated on protein level in cell lines.

The candidate genes were shown to be similarly regulated on RNA level after treatment with Dasatinib or Nilotinib. Although Dasatinib is much less specific than Nilotinib, both drugs influence Bcr-Abl positive cells in the same manner. Including the finding, that drug treatment has only minor effects on Bcr-Abl negative cells, it is very likely, that these genes can be used as potent markers for Bcr-Abl activity.

This list of genes consists of very interesting candidates, including Egr1, CD69, IL10RA, Pim1, IFIT1, SOCS1, SOCS3, p57Kip2 and others that have been previously linked to different types of cancer or even to Bcr-Abl signaling.

5.1.1.1. Egr1

Surprisingly, the most prominently down-regulated gene upon inhibition of Bcr-Abl was Egr1, Early growth response protein 1. This zinc-finger transcription factor is supposed to be a tumor suppressor gene, its expression was reported to be reduced in many different types of tumors [20]. Fukada and Tonkast reported in 2001 that Bcr-Abl down-regulates Egr1 expression [21]. This is in contrast to our data, where drug-dependent Bcr-Abl inhibition leads to down-regulation of Egr1.

Stuart et al. published in 2005, that Egr1 expression is induced by the active c-Abl kinase [22]. This is in line with our results, Bcr-Abl and c-Abl could similarly affect Egr1 expression.

5.1.1.2. Pim-kinases and SOCS proteins

Pim1 and Pim2 expression was reduced to 17% and 44% in the microarray experiment respectively. Pim1 down-regulation was validated by qRT-PCR. Down-regulation of Pim1 after drug treatment matches very well with the literature. Pim-kinases were reported to act as proto-oncogenes. The phosphorylation targets of the Pim1 and its close relatives Pim2 and Pim3 are involved in the regulation of cell cycle and apoptosis. Amongst others, Pim-kinases act on Bcr-Xs, Cdc25A and NFATc and drives the cell towards cell cycle progression. Over-expression and mutations have been found in a number of different tumors including lymphomas, prostate cancer, oral tumors and also CML. Pim1 expression

is induced by Bcr-Abl activated STAT5 [23]. Chen et al. recently reported that Pim1 and Pim2 are down-regulated in Imatinib treated v-Abl positive cells. They also showed higher SOCS1 turnover and subsequent lower SOCS1 levels in Pim1 and Pim2 deficient mice. Furthermore, the SOCS proteins have been shown to be substrates of Pim1. Phosphorylation of SOCS1 and SOCS3 lead to the inhibition of these proteins which in turn blocks their negative influence on the JAK - STAT pathway [23, 24]. In the microarray experiment SOCS1 and SOCS3 were down-regulated to 14% and 19% respectively after drug treatment. SOCS2 was reduced to 33%. Down-regulation of SOCS1 and SOCS3 after cell treatment with Dasatinib and Nilotinib could be confirmed by qRT-PCR. Further investigation of the Pim-kinases and the SOCS-Proteins and their eligibility as marker proteins should be performed.

5.1.1.3. MicroRNA 21

MicroRNA 21 was shown to be down-regulated upon Bcr-Abl inhibition. This microRNA has already been extensively studied. It has been reported to be over-expressed in tumor tissues and it down-regulates tumor suppressor genes like TPM1 and PTEN [25, 26]. This is in line with our results. If miR21 up-regulation is a consequence of Bcr-Abl activity, this microRNA could play an important role in the development of CML.

5.1.2. Candidate investigation on protein level

Three candidates have been further studied on protein level.

5.1.2.1. p57 Kip2

The p57 Kip2 protein is also called Cyclin-dependent kinase inhibitor 1C. Located to the nucleus, it is a binding inhibitor of several G1 cyclin/Cdk complexes and acts a negative regulator of proliferation. It is suspected to be a potent tumor suppressor gene and has already been related to the Beckwith-Wiedemann syndrome and sporadic cancers [27]. These properties make it a potent marker for Bcr-Abl activity.

p57 Kip2 was shown to be 3,5-fold up-regulated on RNA level. On protein level, it was not possible to observe up-regulation after treatment with Dasatinib or Nilotinib for 3 to 24 h. It may be a stable protein with a very low turnover rate. The translation of mRNA into protein may occur very slowly, that up-regulation is not detectable even after 24 h of drug treatment.

This protein is not a suitable marker for Bcr-Abl activity.

5.1.2.2. Regulation of CD69

The second candidate, that was studied on protein level is CD69. This type II integral membrane protein appears to be the earliest inducible surface-glycoprotein during lymphoid activation in T- and B-cells. Upon several stimuli it is inducible in T-cells, B-cells, neutrophils and eosinophils. Several other cell types of the immune system, including bone marrow myeloid precursors, constitutively express CD69. CD69 acts as a signal transduction molecule and is able to induce expression of c-MYC and c-FOS. It is therefore supposed to play a role in lymphocytic proliferation. Binding partners and exact function remain unrevealed [28, 29].

CD69 was down-regulated to 2% expression on RNA level upon drug treatment. Flow cytometry analysis of drug treated K562 cells showed that the surface expression of CD69 is down-regulated to 1/3 upon 16 h treatment with Dasatinib or Nilotinib while no effect could be observed for U937 cells.

Other cell lines or primary cells were not investigated yet. Testing UT7 p210 cells for down-regulation of the protein after drug treatment turned out to be difficult. Induction of Bcr-Abl leads to a modification of size and shape in UT7 cells. UT7 p210 cells again adopt the structure of UT7 par cells. This alteration leads to problems in gating the comparable populations in flow cytometry analysis.

Flow cytometry analysis of primary cells would maybe require a prior sorting of cells and certainly would have to include multiple stainings to distinguish between different cell types.

5.1.2.3. Regulation of Interleukin-8

The cytokine IL8 is expressed by various cell types including monocytes in response to inflammatory stimuli (IL-1, tumor necrosis factor) and functions as chemoattractant for granulocytes. It is also a potent angiogenic factor [1, 30].

IL8 transcription could be shown to be down-regulated in Dasatinib and Nilotinib treated K562 cells to 2 - 10% after 3 h of drug treatment. On protein level, IL8 was also observed to be down-regulated in K562 cells as well as in UT7 p210 cells upon inhibition of Bcr-Abl with Dasatinib and Nilotinib over a period of 24 h. This experimental setup only gives information about the amount of secreted IL8, values for the intracellular IL8 protein levels can not be determined using ELISA. Due to the results on RNA level it is very likely, that not only IL8 secretion but also translation is reduced when Bcr-Abl in inhibited. The drug treatment did not influence the secretion of IL8 in the control cell lines U937 and UT7 par.

Preliminary data could also be achieved for changes of IL8 levels in serum samples of CML patients after onset of Imatinib therapy. It seems as if Imatinib therapy has a similar effect on IL8 secretion in CML patients as Dasatinib and Nilotinib have in cell lines. Elevated IL8 transcription and secretion from myeloblasts derived from $CD34^+$ cells of CML patients have already been reported, the effect of drug therapy on the cells was not tested [31].

5.2. Role of Bcr-Abl core interactors in Bcr-Abl signaling

So far, the signaling of Bcr-Abl is only poorly resolved. Direct interactors of Bcr-Abl are known, but it is not sure if the list is complete. Bcr-Abl either phosphorylates its direct interactors or the binding partners of the core interactors which in turn leads to the activation of a number of important signaling pathways in the cell and to the alteration of the expression pattern of the cell, influencing the cell cycle in an anti-apoptotic and proliferation inducing manner. In the presence of Bcr-Abl in a cell, a number of important signal transduction cascades are active. One pathway may be targeted by more than one core interactor of Bcr-Abl. It is

not clear, which interaction partners of Bcr-Abl are crucial and necessary for the cancerous signaling.

Selective inhibition of close downstream targets of Bcr-Abl could improve treatment of CML and permit better therapeutic achievements in case of drug insensitive Bcr-Abl mutations, but requires detailed information about mechanisms of Bcr-Abl signaling.

By performing single knock-down experiments of known Bcr-Abl core interactor proteins in Bcr-Abl positive cells and analysis of cell growth, critical signaling partners of Bcr-Abl should have been identified. Experiments were performed in K562 cells and UT7 p210 cells, knock-downs efficiencies were between 60% and 90%. Complete elimination of the target proteins was not achieved.

Growth impairment could not be observed for any knock-down. Several reasons may explain these results. The reduction of the copy number may not be sufficient to impair or decrease the signal strength. The remaining copies of the target protein may be enough for sufficient interaction and maintaining the Bcr-Abl signal transduction. Complete removal of the protein would then be necessary to provoke a phenotypic effect.

Another possible explanation is, that the elimination of one single core interactor protein does not impair Bcr-Abl signaling. Other activation routes may overcome the missing signal to keep the critical signaling cascades active. In Bcr-Abl positive cells, various important signaling pathways are active and the disruption of one single activation mechanism may not be enough to reduce the growth and proliferation signal. This could be resolved by performing multiple knock-downs in the cell, but the number of possible combinations is high.

An additional possibility is, that the partial elimination of one core interactor only causes a minor effect in cell proliferation which was not detected by the methods used for the read-out. Growth impairment was only determined by cell counting. More sensitive read-out methods may be necessary to detect the effect. For the knock-down of the core interactors Sts-1 and Cbl an XTT cell proliferation assay was performed but influences on cell growth could not be observed.

Some of the core interactors are suspected to influence Bcr-Abl in a positive manner. The interaction between Bcr-Abl and the core interactor causes forwarding

or amplification of the signal. Some components of the core complex are suspected to be rather negative regulators of the Bcr-Abl signal. The phophatase Sts1, the E3 ubiquitin ligase Cbl and the inositol phosphatase Ship2 are very likely to negatively influence the Bcr-Abl signaling by interfering with the Bcr-Abl signal transducers or inactivation of those. If this is the case, knock-down of the proteins should boost the proliferation of the cells, which could not be observed either.

5.2.1. Suggestions for continuation of the project

Interleukin-8 is planed to be further investigated in serum from CML patients. Statistically significant statements absolutely require expansion of the study. Besides testing a higher number of serum samples from CML patients, also Ph^+ ALL patient material will be analyzed. As controls, the study will include serum samples from patients suffering from other myeloproliferative diseases, samples from patients suffering from other types of leukemia and of course from healthy donors. IL8 can rise quickly due to a minor infection, as it is an important mediator of inflammation. Therefore, it is important to know the state of health of the donors at the timepoint of blood collection.

Further studies on CD69 in other cell lines and in primary bone marrow cells would be necessary to proof the availability of CD69 as marker for Bcr-Abl activity in vivo.

Last but not least, many other possible candidates investigated on RNA level should definitely be studied on protein level. This could not only make them available as marker proteins for Bcr-Abl activity, but it could also help to reveal and understand the signaling network of Bcr-Abl. Interesting candidates are Pim1 and the SOCS proteins. Regulation of miR21 and its role in the development of CML could also be extensively studied. Investigation of similar mode of action as shown for other types of cancer could help to elucidate another mechanism of Bcr-Abl action.

The other part of the project could be pursued by improving the knock-down efficiencies and performance of cell proliferation assays. More sensitive read-out methods for proliferation may be necessary to detect the phenotype of core interactor knock-down. Analysis of combination of knock-down of multiple core

interactors could reveal which core interactors are involved in the same signaling pathways. One could also test, if the knock-down of the Bcr-Abl interactors influences the expression of the activity markers investigated in the first part of this work.

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A. Appendix - Supplementary figures

Table A.1: *siRNA constructs*

BAF7 (sequence specific to Bcr-Abl breakpoint region)
sense: 5‘-CAGAGUUCAA | AAGCCCUUCAGdTdT-3‘
antisense: 3‘-dTdTGUCUCAAGUU | UUCGGGAAGUC-5‘

BAF8 (missmatch control)
sense: 5’-CAGUGUUCAU | AAGCCGUUCAGdTdT-3’
antisense: 3‘-dTdTGUCACAAGUA | UUCGGCAAGUC-5‘

Table A.2: *qRT-PCR primer*

Gene	Forward primer	Reverse Primer
Egr1	TGACCGCAGAGTCTTTTCCT	TGGGTTGGTCATGCTCACTA
CD69	CATGAAGGGTCCTTCCAAGT	TGATGGCATTGAGAATGTGTATT
IL8	GGACAAGAGCCAGGAAGAAA	AAACTGCACCTTCACACAGAG
IL10RA	TGGACCTGTACCACAGCAAT	GGTTCACACTGCCAACTGTC
CISH	TTCAGGGACCTCGTCCTTT	GGCTATGCACAGCAGATCCT
SPRY4	AGCCTGTATTGAGCGGTTTG	GGTCAATGGGTAGGATGGTG
PHLDA1	CAAGGAGATCGACTTTCGGT	ATGGCCTGACGATTCTTGTA
GDF15	AAGATTCGAACACCGACCTC	AGAGATACGCAGGTGCAGGT
MAFF	TCTGTGGATCCCCTATCCAG	CTTCTGCTTCTGCAGCTCCT
HECW2	GGGAGGAAGCTGACTTTCAC	TGAAGAACTCTGGGCTGATG
SOCS1	CTGGGATGCCGTGTTATTTT	ACCCCCTCAAGAGGTGAGAA
SLC2A3	CAAGGATGCAGGTGTTCAAG	AGTCCTTCTTCCTGCCCTTT
HHEX	GGGCAAACCTCTACTCTGGA	GCGTCTCGAATTTCTTCTCC
APOL4	CTGGAGGACGTGTCTGGTTA	CCTCCTCCAAGGGATCTTC
PIM1	CTGCTCAAGGACACCGTCTA	TGGTAGCGGATCCACTCTG
CHAC1	GGAGACACCTTCCATCGG	TCATGATCTTCAAGGAGCGT
BHLHB2	GCAAGGAGACCTACAAATTGC	TCGGGTAGGAGATCCTTCAG
BCL2L1	GGATGGCCACTTACCTGAAT	CTGCTGCATTGTTCCCATAG
SOCS3	GAGGATCCTGGTGACATGCT	CAAATGTTGCTTCCCCCTTA
ATF3	GTGCCTGCAGAAAGAGTCG	GAGCTCCTCAATCTGAGCCT
ETV5	TTTATGGTCCCAGGGAAATC	CTTCAGAATCGTGAGCCAGA
STC2	AGAATACAGCGGAGATCCA	AACATTCAAACACGCCACAC
AMIGO2	GAAGTCGGAAGGTGCAAGAG	TGAGTCTCTTCCCGGTGTCT
ITPR1	AGGCCGAAGATACCAAGATG	ATCCCTGGGTTGAGAAACAG
ERMAP	CGGAGGTGGACAATCTTCTT	GCTCTTCTCCAGCCTGAGTT
TMEM56	GTGGTCCATCACTTGCAAAC	TTTGTCACCAATCACTTTCCA
FAM46B	ACCTGAGTGGGCTGAGCT	ATGTAGTCCCTGCTCCTCCA
IFIT1	TCTCAGAGGAGCCTGGCTAA	TCAGGCATTTCATCGTCATC
LGR4	CCACACTTGGGCCAATAACT	TGCTGCTAAGGCTTCTTTCA
p57Kip2	GAGATCAGCGCCTGAGAAGT	GGGCTCTTTGGGCTCTAAAT
CYP3A5	TAGGCCCAGTGGGATTTATG	CATCTCCATACTGGGCAATG
STS1	ACAAATGGTCCGAAAGATCC	TGGGTAAGAGGAAGGATTGG
SHIP2	AAGGCCCTACAGGACATGAG	CTTCCCAATCTTGGTCAGGT
GRB2	CTCCCTCTCTGTCAAGTTTGG	GTACTTCCCGGCTCCATCT
SHC1	TGATCCCTCCTATGTCAACG	ATCTTCGAAGGGCTTCATGT
CRK2	CCCACTACATCATCAACAGCA	TCCAGTAAAGCAGGCAATGA
p85alpha	TCATTGTCGGATACAGGCAT	GGTGGTTTAGGAGGCAGTGT
p85beta	AGATGGGCACTATGGCTTCT	TCTGGTCCTGCTGGTATTTG
CBL	ATTCATTCACAAACCTGGCA	TCTGGAGAATGTTCCCATCA

Table A.4: *Expression changes of candidate genes in K562 cells measured by microarray and qRT-PCR and in U937 cells measured by qRT-PCR*

Candidate	microarray	K562 Dasatinib	st. dev. K562 Dasatinib	K562 Nilotinib	st. dev. K562 Nilotinib	U937 Dasatinib	st. dev. U937 Dasatinib	U937 Nilotinib	st. dev. U937 Nilotinib
EGR1	0,011	0,032	0,038	0,004	0,005	0,089	0,012	0,075	0,051
CD69	0,027	0,027	0,010	0,035	0,004	0,805	0,047	0,879	0,878
IL8	0,060	0,026	0,004	0,146	0,059	6,380	2,058	14,753	4,069
IL10RA	0,061	0,036	0,007	0,030	0,009	1,730	0,412	1,432	0,140
CISH	0,064	0,027	0,007	0,044	0,018	0,181	0,040	0,640	0,109
SPRY4	0,085	0,043	0,038	0,003	0,000				
PHLDA1	0,108	0,076	0,006	0,113	0,001				
GDF15	0,111	0,045	0,017	0,060	0,001				
MAFF	0,119	0,105	0,094	0,180	0,041				
HECW2	0,123	1,203	1,040	0,844	0,095				
SOCS1	0,141	0,087	0,087	0,244	0,206				
MYC	0,143								
SLC2A3	0,144	0,102	0,048	0,162	0,065				
HHEX	0,148	0,092	0,012	0,136	0,050	1,267	0,337	2,558	0,523
APOL4	0,169	0,570	0,226	0,330	0,079				
PIM1	0,172	0,106	0,014	0,086	0,003	0,476	0,007	1,654	0,218
CHAC1	0,192	0,114	0,029	0,279	0,080				
BHLHB2	0,193	0,107	0,004	0,161	0,056				
BCL2L1	0,195	0,152	0,032	0,266	0,143				
SOCS3	0,196	0,039	0,001	0,041	0,005	0,623	0,213	0,375	0,352
ATF3	0,210	0,095	0,061	0,137	0,008				
ETV5	0,226								
STC2	0,239	0,168	0,101	0,256	0,070				
AMIGO2	0,241	0,144	0,029	0,218	0,062	3,353	0,410	1,852	1,623
ITPR1	0,274								
miRNA21	0,282								
ERMAP	2,404	2,819	0,576	3,812	0,149	0,701	0,051	1,382	0,375
TMEM56	2,552	1,623	0,409	3,046	1,489	1,981	0,097	3,192	0,637
FAM46B	3,002	3,723	0,491	7,146	0,560				
IFIT1	3,292	16,728	1,800	44,255	6,484	0,599	0,023	1,054	0,524
LGR4	3,349								
p57Kip2	3,701	3,319	1,102	3,802	0,261	0,726	0,075	1,238	0,944
CYP3A5	5,509	3,343	0,587	6,825	0,334	2,690	1,384	3,302	1,851

Table A.6: *Influence of drug treatment on IL8 secretion of K562 and U937 cells*

Cell line	Time of treatment	Type of treatment	IL8 (pg /1*10^6 cells/ml)	standard deviation
K562	3h	no treatment	172,1	16,2
		Dasatinib	150,0	2,7
		Nilotinib	155,6	0,1
	9h	no treatment	396,6	84,3
		Dasatinib	203,6	20,6
		Nilotinib	220,1	17,7
	24h	no treatment	647,8	156,7
		Dasatinib	268,5	15,6
		Nilotinib	271,6	12,9
U937	3h	no treatment	33,9	13,9
		Dasatinib	32,8	21,4
		Nilotinib	27,1	14,9
	9h	no treatment	61,6	33,3
		Dasatinib	47,2	19,3
		Nilotinib	33,9	11,4
	24h	no treatment	31,4	7,8
		Dasatinib	26,1	6,6
		Nilotinib	28,5	2,0

Table A.7: *Influence of drug treatment on IL8 secretion of UT7 p210 and UT7 par cells*

Cell line	Time of treatment	Type of treatment	IL8(pg/1*10^6 cells/ml)	standard deviation
UT7 p210	3h	no treatment	611,3	21,8
		Dasatinib	150,3	0,2
		Nilotinib	221,1	0,6
	9h	no treatment	841,2	9,4
		Dasatinib	276,4	10,6
		Nilotinib	369,6	8,1
	24h	no treatment	1057,0	20,4
		Dasatinib	355,5	7,3
		Nilotinib	471,3	2,7
UT7 par	3h	no treatment	108,0	5,1
		Dasatinib	93,8	31,9
		Nilotinib	66,1	7,1
	9h	no treatment	323,4	15,3
		Dasatinib	261,4	7,6
		Nilotinib	236,6	10,8
	24h	no treatment	494,1	13,4
		Dasatinib	455,0	
		Nilotinib	426,4	12,3

Table A.8: *Influence of Imatinib therapy on IL8 levels in serum of CML patients*

Patient	Time of therapy	IL8 (ng/ml serum)	standard deviation
CML patient 1	diagnosis	3,788	0,750
	1 month	0,053	0,016
	3 months *	0,104	0,047
CML patient 2	diagnosis	1,786	0,108
	1 month	1,585	0,010
	3 months	0,086	0,004
	12months	1,527	0,035
CML patient 3	diagnosis	0,576	0,026
	diagnosis	0,361	0,011
	1 month	0,125	0,013
	3 months	0,370	0,013
	24 months *	0,128	0,011
CML patient 4	diagnosis	0,535	0,026
	1 month	0,179	0,013
	3 months *	0,059	0,006
healthy donors	ctr	0,108	0,008
	ctr	0,058	0,002
* complete cytogenetic response			

Curriculum Vitae

CONTACT INFORMATION

Name:	Agnes Gstöttenbauer
Date of Birth:	28.12.1983
Address:	Bürgerstraße 3/5
Zip, City:	A-4209 Engerwitzdorf
Telephone:	+43-650-246 37 01
Email:	agnes.gstoettenbauer@gmail.com

EDUCATION

February 2007 - February 2008	Diploma Thesis CeMM, Austrian Academy of Sciences
since March 2003	Universtiy of Vienna Student of Molecular Biology
October 2002 - February 2003	Paris Lodron University Salzburg Student of Biology
12. June 2002	Matura Europagymnasium Auhof, Linz

Agnes Gstöttenbauer