Ludwig Boltzmann Cluster Oncology LB-CO
Progress Report 2011

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Following the work plan and project aims of the LB-CO, substantial new data and results have been generated during the 4th project-year (2011). New stem cell markers and targets have been identified, new insights into stem cell-niche interactions have been accumulated, and the role and function of several targets expressed in leukemic stem cells, have been determined. In several instances, we were also able to study the effects of various targeted drugs on leukemic stem- and progenitor cells.

1. Overview of the LB-CO
1.1. Introduction and Aims

During the past decade numerous studies have shown that most if not all leukemias are composed of two different cell fractions, a bulk population of leukemic cells with limited capacity to divide, and a second smaller cell population exhibiting the capacity of unlimited growth and self-renewal, the so-called leukemic stem cells (LSC). This hypothesis is now widely accepted and predicts that minimal residual disease, relapsing disease, and thus all relevant disease components, contain LSC, and that any therapy is curative only when eliminating most or all LSC in a given leukemia. During the past few years, more and more data have shown that leukemia stem cell fractions represent heterogeneous populations of cells, reflecting plasticity and genetic instability in the pool(s) of LSC. So far, however, little is known about the expression of relevant targets in LSC and LSC-derived subclones (Figure 1). From these considerations it seems important to learn more about target expression profiles of LSC and about responses of these cells to targeted drugs, conventional drugs, and immunotherapy.

The aims of the current project are to identify and characterize LSC in various human leukemias, to define target expression profiles for these cells, and to examine the effects of various conventional and targeted drugs on growth and survival of LSC. Drugs and markers were selected based on our previous results and the available literature. The long-term goal of the project is to establish new improved (potentially curative) therapies for human leukemias by applying drugs that recognize and eliminate LSC.
Figure 1
Expression of cell surface and cytoplasmic target antigens in leukemic cells. Many therapeutic targets have been identified in bulk leukemic cells; however, little is known about expression of these molecular targets in leukemic stem cells (LSC).

General Aims in LB-CO Projects:

- Identification and phenotypic characterization of LSC in various leukemias

- Characterization of target expression profiles in LSC

- Delineation of stem cell plasticity and stem cell resistance: relationship to genetic instability and characterization of relevant stem cell subclones

- Targeting of leukemic stem cells using specific targeted drugs

- Evaluation of interactions between neoplastic stem cells and the SC-Niche

- Evaluation of effects of endogenous immune-mediators (cytokines) and death-regulators on growth and survival of neoplastic stem cells
1.2. Budget Plan

The budget plan was established in cooperation with the LB society. Budget was mainly used to employ personnel and to purchase consumables. Part of the budget was used to organize meetings, to invite authorities in the field or to send Postdocs to conferences and meetings. The total LB-CO budget scheduled for 2011 was 823,000.€. A detailed summary of the LB-CO budget in 2011 is provided in a separate file.

1.3. Partners and Internal Structure of the Cluster

Partners
As in previous years, two scientific partner institutes were involved in the LB-CO in 2011, namely the Hanusch Hospital and the Medical University of Vienna.

Project Groups and Scientists
Within the LB-CO, 4 different project lines (PLs) were initially established: a) myeloid neoplasms, b) lymphoid neoplasms, c) solid tumors, and d) malignant melanoma and other skin neoplasms/mastocytosis. Group ‘a’ also worked on normal stem cells and myelodysplastic syndromes (MDS). According to the evaluation report 2010 and following its recommendations, we have finalized all projects in PL ‘c’ and all melanoma projects in PL ‘d’ in 2011. These projects were replaced by projects focusing on human leukemias including mast cell leukemia, and by research focusing on stem cell-niche interactions. The following researchers were employed in 2011: Heidrun Karlic (MDS and AML), Harald Herrmann (AML, CML), Sabine Cerny-Reiterer (ALL), Barbara Peter (mast cell leukemia) and Emir Hadzijusufovic (myeloid and mast cell leukemias). The group organized weekly scientific meetings (2 hours), one Week Start Meeting per week (Monday, 10:00), and a Lecture Series (Friday 16:00). The group was also involved and participated in several stem cell meetings and key hematology congresses.

Administration
Administration was coordinated by the administrative head, Prof. Dr. Thomas Grunt and our secretary, Sabine Sonnleitner, who handled logistic, administrative, and technical issues in the LB-CO.

Core Facility Groups
All core facility (CF) groups established in cooperation with the Medical University of Vienna, were also used in the 4th project year. CF-Platforms (PF) included a stem cell sort PF (employing a high speed sorter), a gene chip PF (including bioinformatics, proteomics, and deep sequencing facilities), a NOD/SCID (NSG) mouse PF (in cooperation with the Veterinary University of Vienna), and a clinical PF (including a biobanking system and several local registries for data capture and data calculations). All these CF PFs were essential for the progress in our LB-CO projects in the year 2011.
1.4. Scientific Advisory Board (SAB)

Based on discussions with the Society and the recommendation of the evaluation report, an external SAB was established. The first SAB meeting was scheduled for March 2, 2012. In addition, the LB-CO Cluster will maintain its local SAB with the same panel of experts that was in charge in the previous project phase.

1.5. Personnel and Career Development

The following Scientists were employed via the LBG in the LB-CO Cluster in 2011:
Heidrun Karlic  Hanusch Hospital  01-12/2011
Harald Herrmann  Medical University of Vienna  01-12/2011
Sabine Cerny-Reiterer  Medical University of Vienna  10-12/2011
Barbara Peter  Medical University of Vienna  06-12/2011
Emir Hadzijusufovic  Medical University of Vienna  10-12/2011

Career development steps: Harald Herrmann will start a clinical career at the Medical University of Vienna in March 2012. Heidrun Karlic acquired Professorship in 2010. The other group members have started to work in the LB-CO cluster in 2011, and will work on their scientific career in this environment. Within the group of non-LBG-employed LB-CO colleagues, one application for an Associate Professorship should be mentioned.

1.6. Infrastructure

The infrastructure in 2011 included three labs (3x30 m²) dedicated to LSC research at the University, several other labs of scientists participating in the LB-CO network, as well as a lab at the Hanusch Hospital (HK). In addition, one office for our secretary (Sabine Sonnleitner) as well as core facility rooms were made available and were used to run LB-CO projects. All in all, the labs and infrastructure shared by partners as well as the scientific environment provided optimal conditions for our LB-CO projects in 2011.

1.7. Scientific Highlights

Among several scientific highlights and results in the LB-CO the following outstanding observations of 2011 should be mentioned: 1. Identification of DPPIV (CD26) as an SDF-1-degrading and disease-specific key stem cell marker in CML, 2. The phenotypic characterization of CD34+/CD38- SC in Ph+ and Ph- ALL, 3. The characterization of novel immature mast cell progenitor cell lines and KIT mutant-bearing subclones, and 4. The phenotypic characterization of CD34+/CD38- SC in patients with indolent (ISM) and aggressive systemic mastocytosis (ASM). Published highlights were the detection of CD33 on CML stem cells, HO-1/Hsp32 in AML stem cells, effects of the BRD4-blocking drug JQ1 on AML precursor cells, and the effects of novel KIT tyrosine kinase inhibitors (TKI) on growth of neoplastic mast cells in advanced systemic mastocytosis.
1.8. Public Relation

The Mission of the LB-CO Cluster is to increase awareness and to gain knowledge in the field of cancer/leukemic stem cells, to establish pathogenetic and target-related concepts around these cells, and to establish stem cell-eradicating approaches, with the ultimate aim to improve curative anti-cancer therapy. The LB-CO Cluster Team was able to transfer its Mission-Intention to the Public in 2011 through publications and participation in meetings organized by the LB Society. In addition, the cluster was essentially involved in the “Year 2011 Working Conference on Cancer Stem Cells” in Vienna.

2. Results obtained in the LB-CO Cluster in 2011

2.1. Results obtained in Individual Projects

2.1.1. Epigenetic Regulation of FAS in MDS, AML, and MCL

During the first 3 years of our LB-CO project, we were able to show that FAS mRNA- and protein expression is upregulated in low risk MDS (compared to normal bone marrow) and that FAS becomes hypermethylated and downregulated during disease progression to high risk MDS (with blast excess) or AML. We were also able to show that the 2 demethylating agents used to treat MDS patients, 5-azacytidine and decitabine, induce FAS (re)expression and apoptosis in AML cells. During the 4th project year, we have extended our investigations in this project line. The following results were obtained:

In a first step, we confirmed that FAS (CD95) is also expressed on neoplastic stem cell-enriched fractions in patients with MDS. In these experiments, we found that the levels of FAS (CD95) on CD34+/CD38- and CD34+/CD38+ cells are higher in patients with low risk MDS than in patients with high risk MDS or AML (Figure 2).

![Figure 2](image_url)

Expression of FAS (CD95) on stem- and progenitor cells in MDS and AML

Bone marrow MNC were obtained from patients with low risk MDS (low blast count) (n=7), high risk MDS (RAEB) or secondary AML (n=8). Expression of FAS on CD34+/CD38- and CD34+/CD38+ stem- and progenitor cells was determined by multi-color flow cytometry. Results show the staining index (MFI compared to control) and represent the mean±S.D. of all donors examined. Asterisk: p<0.05.
We also screened for expression of FAS and effects of demethylating agents in other myeloid neoplasms. Among these, we found that drug-induced FAS-expression is also seen and is also accompanied by apoptosis in MCL. Notably, both 5-aza-cytidine and decitabine were found to inhibit the growth and to induce apoptosis in primary neoplastic mast cells as well as in the human mast cell leukemia cell lines HMC-1.1 and HMC-1.2.

In a next step, we asked whether drug-induced FAS-re-expression is a critical event responsible for drug-induced apoptosis. To address this question, we applied an siRNA against FAS. After transfection, neither 5-aza-cytidine nor decitabine was able to induce FAS expression. Moreover, FAS-siRNA-transfected cells were found to be resistant against the apoptosis-inducing effects of both demethylating agents. These effects of FAS siRNA were observed in all AML cell lines tested and in both MCL cell lines (Figure 3). These observations suggest that FAS is a critical molecule in drug-induced apoptosis.

![Image](image1.png)

**Figure 3**
Effects of FAS siRNA on drug-effects in HMC-1 cells
A: HMC-1.2 cells were kept in control medium (untransfected) or were transfected with siRNA against FAS (200 nM) using lipofectin. Cells were then incubated in control medium (open black histograms), 5-Aza-cytidine, or Decitabine (each 5 μM, grey histograms) at 37°C for 30 hours. Thereafter, CD95 expression was analyzed by flow cytometry. Antibody-staining was controlled by isotype-matched antibodies (grey-lined open histograms). B: After transfection and exposure to 5-Aza-cytidine or Decitabine (each 5 μM, 30 hours), active caspase-3 staining was performed by flow cytometry. Results show the percentage of active caspase-3-positive cells.

Finally, we examined the effects of the FAS ligand on growth and survival of neoplastic cells in MCL. In these experiments, we found that the FAS ligand strongly promotes apoptosis-inducing effects of 5-Aza-cytidine and Decitabine in MCL. By contrast, in the absence of demethylating agents, FAS-ligand did not induce apoptosis in MCL cells. We also examined whether FAS-ligand and the demethylating agents tested would exert cooperative anti-neoplastic effects in AML and CML, and in other myeloid neoplasms. However, in all other cell line models examined, including 3 AML cell lines (HL60, U937, KG1) and 2 chronic myeloid leukemia (CML) cell lines (K562, KU812), no cooperative effects of the drug combination treatments tested were seen. Thus, the cooperative effect of FAS ligand seems to be specific to MCL.
2.1.2. Evaluation of Regulation and Function of Interleukin-Receptors on Neoplastic Stem Cells in Myeloid Neoplasms

During the past 4 years of the LB-CO, a total number of 534 samples (BM or PB) were analyzed. Diagnoses were AML (n=184), MDS (n=68), myeloproliferative neoplasms (MPN, n=35), and chronic myeloid leukemia (CML, n=148). In addition, 89 cases of reactive/normal bone marrow and 18 cord blood samples were included. Expression of surface molecules on CD34+ subfractions (CD34+/CD38+, CD34+/CD38-) and CD34- cells were analyzed by multicolor flow cytometry. In a group of patients, CD34+/CD38+ cells, CD34+/CD38- cells, and CD34- cells were sorted from mononuclear cells (MNC) to near homogeneity (>98% purity) by high-speed sorting. Isolated cells were subjected to mRNA isolation, qPCR, ³H-thymidine incorporation experiments (effects of cytokine-ligands and drugs), or gene chip profiling. Whenever possible, FISH was performed to confirm the leukemic nature of sorted cells. Spontaneous and drug-induced apoptosis of AML stem cells was determined by combined staining for surface markers and AnnexinV. A number of AML-related myeloid cell lines were examined, including KG1, HL60, U937, and MO7e. A KG1 subclone containing a CD34+/CD38- and a CD34+/CD38+ subfraction was also examined. Results obtained from gene chip experiments were confirmed by real time PCR and flow cytometry and/or immunocytochemistry. A number of novel potential stem cell markers were identified.

In the 4th year of our LB-CO project, we focussed on cytokine receptors that may play a role in immunomodulation and growth of leukemic stem cells (LSC) in AML and CML, such as the IL-2R or the IL-3R. In these experiments, we were able to show that IL-2RA CD25 is selectively expressed on LSC in CML (all donors) and AML (20-30% of all donors), but not on normal (resting) BM stem cells. We were also able to show that LSC in AML and CML express not only the IL-3RA but also the common beta chain of IL-3R, IL-5R, and GM-CSFR. However, whereas LSC expressed substantial amounts of IL-3RA, these cells did not express substantial amounts of IL-5RA or GM-CSFRA. We also examined LSC for expression of the high affinity IL-2R. However, although analyzed extensively, we were unable to detect IL-2RB (CD122) in LSC. Next, we screened for expression of histamine receptors (HR) on LSC. As determined by flow cytometry, the HR4 was found to be expressed on LSC in a few patients with AML. However, in most cases tested, AML LSC and CML LSC did not express substantial amounts of HR1, HR2, or HR4. In a next step, we examined the effects of various LSC-interacting cytokines and histamine on IL-receptor expression and ROS expression in neoplastic cells. We found that histamine slightly upregulates ROS levels in AML and CML cells, and in the CML cell lines KU812 and K562, whereas no effects were seen with IL-2 or the other cytokines tested. Finally, we asked whether leukemia-specific oncogenic signaling pathways are involved in aberrant expression of CD25/IL-2RA. We found that BCR/ABL-blockers (imatinib, nilotinib) as well as STAT5-targeting drugs substantially downregulate expression of CD25 on CML cell lines as well as on primary CML LSC.
2.1.3. Identification of CD26 as CML LSC-Specific Target-Enzymes disrupting SDF-1-CXCR4-interactions in the SC Niche

a. Identification of CD26 as a novel specific stem cell marker on CML LSC
During the 4th project year, we screened specifically for LSC antigens that may play an important or even decisive role in LSC-niche interactions in CML. Of all markers identified by gene chip screening and antibody-based screening, the SDF-1-degrading surface enzyme Dipeptidyl-Peptidase IV (DPPIV=CD26) was identified as the most specific and the most promising marker-antigen. In newly diagnosed patients with CML with high leukocyte counts, most of the CD45+/CD34+/Lin-/CD38- cells in the BM were found to co-express CD26 by flow cytometry (Figure 3). When purified by cell-sorting (purity >99%), these CD34+/CD38-/CD26+ cells were found to express BCR/ABL by FISH and qPCR, whereas CD34+/CD38-/CD26- cells (presumably normal SC) obtained from the same patients (low leukocyte counts) were found to lack BCR/ABL (Figure 4).

![Figure 4](image)

Identification of CD26 as a specific marker for CD34+/CD34-/Lin- stem cells in CML
A: Bone marrow MNC obtained from a patient with CML (left panels) and from a non-leukemic control case (right panels) were stained by multi-color flow cytometry using a mAb to CD26. CD45+/CD34+/CD38- CML cells (MNC) were found to stain positive for CD26, whereas the CD34+/CD38- cells in the control sample stained negative for CD26. B: In a few patients with early phase CML (low WBC), two fractions of CD34+/CD38- cells were detected, a CD26+ and a CD26- subfraction. Only the CD26+ fractions were found to be BCR/ABL+ cells by FISH.
In the peripheral blood (PB) of patients with freshly diagnosed CML, most of the CD34+/CD38- cells were found to co-express CD26, whereas CD34+/CD38-/CD26- (normal) stem cells were usually not detectable in the PB of these patients. CD26 was also found to be an LSC-specific marker in that the more mature CD34+/CD38+ BM and PB CML cells were found to lack CD26 (Figure 4). CD26 was neither detectable on CD34+/CD38- BM cells in healthy controls nor on CD34+/CD38- BM cells in patients with other myeloid neoplasms or in the reactive BM (Table 1). To confirm expression of CD26 on CML SC, we applied a number of different CD26 antibodies, all of which produced identical staining reactions with CD34+/CD38- cells. We also confirmed expression of CD26 mRNA in CD34+/CD38- CML cells by qPCR. In more mature hematopoietic cells, the distribution of CD26 in CML patients was identical to that found in healthy controls. Notably, like in healthy controls, blood basophils and a subset of T lymphocytes were found to stain positive for CD26 in CML. In addition, cultured endothelial cells (HUVEC) were found to stain positive for CD26 by flow cytometry.

To demonstrate that the CD26+ progenitor cell fraction indeed contains long-term leukemia-propagating LSC, we injected highly enriched subfractions of CD34+ cells into NSG mice. Whereas CD34+/Lin-/CD26- cells produced multilineage (lymphoid and myeloid) engraftment with BCR/ABL-negative (normal) cells, almost all NSG mice injected with CD34+/Lin-/CD26+ BM cells showed prominent or even selective myeloid (granulocytic/granulomonocytic) engraftment with BCR/ABL+ cells after 16-20 weeks.

b. Expression of other cell surface molecules on CML LSC
We next compared expression of CD26 on CML LSC with expression of other LSC markers. In these experiments, we found that apart from CD26, also other markers are expressed on immature CD34+/CD38- CML LSC. These markers were IL-2RA (CD25), KIT (CD117), IL-3RA (CD123), AC133 (CD133), CXCR-4 (CD184), and IL-1RAP (Table 1). In addition, we confirmed that CD26+ LSC co-express Siglec-3 (CD33) and Pgp-1 (CD44). Of all markers tested, only CD25, CD26, and CD133 were found to be rather specific markers of immature CML LSC in that more mature CD34+/CD38+ BM cells did not express the same antigens. By contrast, all other markers including IL-1RAP were also expressed on more mature clonal cells. With regard to normal (non-leukemic) BM SC, CD25, CD26, and IL-1RAP were found to be the most specific LSC markers in CML. By contrast, all other SC-related antigens were also detectable on normal SC (Table 1). When comparing to other leukemias, especially acute myeloid leukemia (AML), only CD26 was identified as a specific CML-LSC antigen. Notably, both CD25 and IL-1RAP were also detectable on LSC in a substantial number of cases with AML, whereas CD26 was usually not detected on LSC in MDS or AML (Table 1).

c. Regulation of expression of CD26 on CML cells
To study the mechanisms of expression of CD26 on CML progenitor cells, we examined the effects of various signal-transduction blockers. Whereas imatinib and STAT5 inhibitors were found to downregulate expression of CD25 on CML LSC as well as on the CML cell line KU812, no effects of these drugs on expression of CD26 were seen. Moreover, we were unable to detect major effects of RAS inhibitors, MEK inhibitors,
PI3-kinase inhibitors or mTOR inhibitors on expression of CD26 on CML (stem) cells. These data suggest that CD26 expression on CML LSC may be a BCR-ABL-independent event that may even precede the expression of BCR-ABL in LSC, whereas expression of CD25 on CML SC is triggered by BCR-ABL, and probably by STAT5-activity.

Table 1
Expression of Cell Surface Markers and Targets on CD34+/CD38- Stem Cells in various Leukemias and in the Normal Bone Marrow (BM)

<table>
<thead>
<tr>
<th>Marker/Target</th>
<th>CD</th>
<th>CML</th>
<th>AML</th>
<th>MCL</th>
<th>MDS</th>
<th>Normal BM</th>
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<tr>
<td>IL-2RA</td>
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<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DPPIV</td>
<td>26</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Siglec-3</td>
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<td>+</td>
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<tr>
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<tr>
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cβ-ILR, common beta chain of IL-3/GM-CSF/IL-5-R; n.c., not yet clustered; n.t. not tested.

d. Potential Function of CD26 (DPPIV) on CML LSC
We next examined the functional significance of CD26-expression in CML stem cells. As the SDF-1-CXCR4 axis has been implicated in SC-homing, and CD26 is known to cleave SDF-1 to disrupt its function, we asked whether CD26 expression on CML LSC is associated with the well-known migration-defect of CML LSC against SDF-1. In a first step, we confirmed that CD26 on CML stem cells indeed cleaves SDF-1. Then, we expressed CD26 in K562 cells and KU812 cells by lentiviral-mediated gene transfer. Lysates prepared from these CD26+ CML cell lines were found to degrade SDF-1 and to inhibit the migratory response of CXCR4+ leukemic cells to SDF-1. The CD26-targeting
drugs sitagliptin and vildagliptin were found to restore (in part) this migratory response (of CXCR4+ cells) to SDF-1 (Figure 5). Collectively, these data suggest that CD26 may be a functionally important LSC marker in CML, and that CD26 expression facilitates the escape of LSC from the SC niche via disruption of the SDF-1-CXCR4 axis. Currently, we examine the effects of various gliptins on the in vitro growth and migration of CML LSC, and on LSC-homing to BM sites/niches in NSG mice (after either i.v. injection or intramedullary injection). In addition, we plan to establish a clinical trial using gliptins in combination with nilotinib in patients with CML. In a first pilot patient (CML under nilotinib therapy) the levels of BCR/ABL decreased during treatment with saxagliptin.

**Figure 5**

Vildagliptin counteracts CD26-mediated inhibition of SDF-1-induced leukemia cell migration CXCR4+ leukemic cells were induced to transmigrate through a filter-membrane against recombinant SDF-1 (5 ng/ml) in a double-chamber chemotaxis assay. Lysates prepared from CD26-transfected KU812 cells were found to degrade SDF-1 (not shown) and neutralized the migratory effect of the ligand. Addition of vildagliptin, a CD26-targeting drug, reverted (in part) the inhibitory effect of the CD26-containing KU812 lysate.

c. Distribution of SDF-1, CD26, and of CD34+ progenitor cells in BM niches

Whereas many leukemias start to regenerate the BM from the endosteal niche (after e.g. chemotherapy), CD34+ CML progenitors were not found to accumulate in the endosteal region, neither in NSG mice nor in CML patients. These data suggest that CML LSC may not be capable of homing to the SC niche in the same way as AML LSC. We also examined the distribution of SDF-1 and CD26 in the leukemic BM by immunohistochemistry. Whereas CD26 was detected in endothelial cells of larger and smaller blood vessels (positive control), SDF-1 was only detectable in vascular endothelial cells but not in endosteal cells in CML, and the same result was obtained for the normal BM.

All in all, the identification of CD26 as a specific marker and target expressed on LSC in CML may be a major novel finding that could contribute essentially to our understanding the pathogenesis (extramedullary LSC spread) and clinical manifestation of CML, and assist in the isolation and investigation of CML LSC. In addition, targeting of CD26 may be a novel therapeutic approach to restore the disrupted SDF-1-CXCR4 axis in CML.
2.1.4. Expression and role of pSTAT5 in CD34+/CD38- Stem Cells in Myeloid Leukemias and Preleukemic Disorders

Various signal transduction molecules have been implicated in oncoprotein-dependent transformation and expansion of myeloid progenitor cells in various hematologic neoplasms. In myeloid neoplasms, including preleukemic syndromes, the transcription factor STAT5 has recently been described to play an essential role in progenitor cell proliferation and survival. We have recently shown that activated (phosphorylated) STAT5 (pSTAT5) is expressed in the cytoplasm of neoplastic myeloid cells in systemic mastocytosis (SM) and mast cell leukemia (MCL) as well as in patients with CML and AML. We have now extended these investigations to LSC-enriched fractions of leukemic cells and to myeloproliferative disorders, a group of preleukemic condition where JAK2 V617F is considered as an essential upstream regulator and activator of STAT5.

As assessed by immunocytochemistry, primary neoplastic JAK2 V617F+ cells and HEL cells, a JAK2 V617F+ erythroleukemia cell line, expressed phosphorylated (p) STAT5 in their cytoplasm and less abundantly in their nuclei. We next examined expression of pSTAT5 in CD34+/CD38- stem cells in patients with JAK2 V617F+ myeloproliferative neoplasms (MPN) by flow cytometry. In all patients tested (n=9), the CD34+/CD38- stem cells were found to express pSTAT5, whereas CD34+/CD38- stem cells in the normal/reactive BM did not express pSTAT5 (Figure 6).

![Graphs showing expression of pSTAT5 in different cell types](image)

**Figure 6**  
Expression of (cytoplasmic) pSTAT5 in primary CD34+/CD38- stem cells  
Bone marrow cells obtained from a patient with JAK2 V617F+ ET, one with JAK2 V617F+ PMF, one with Ph+ CML (positive control), and one non-leukemic control (Co), were analyzed by multicolor flow cytometry using antibodies against CD34, CD38, and pSTAT5. Before being stained, cells were fixed in 2% formaldehyde and permeabilized using icecold methanol.
To confirm that pSTAT5 is indeed expressed in the cytoplasm of JAK2 V617F-transformed cells, we performed Western blot experiments on nuclear extracts and cytoplasmic extracts of HEL cells. In these experiments we were able to show that substantial amounts of pSTAT5 are expressed in the cytoplasm of HEL cells (Figure 7).

Figure 7
Expression of cytoplasmic pSTAT5 in HEL cells
A: HEL cells were lysed in hypotonic buffer to generate extracts. Cytoplasmic extracts (CE) and nuclear extracts (NE) were prepared by centrifugation. Expression of pSTAT5 was determined by Western blotting (A), and confirmed by densitometry (not shown). B: Immunocytochemical detection of pSTAT5 on cytospin slides using an anti-pSTAT5 antibody and a staining protocol optimized for detection of cytoplasmic pSTAT5 in neoplastic cells.

In a next step, we applied the STAT5-targeting drugs piceatannol and pimozone. As assessed by ³H-thymidine uptake, both drugs were found to inhibit the proliferation of HEL cells and of primary neoplastic cells in a dose-dependent manner (Figure 8). Both drugs were also found to induce apoptosis in HEL cells. Currently, we are examining the effects of siRNAs and a dominant-negative STAT5 construct to confirm that pSTAT5 is a valuable target in JAK2 V617F-transformed cells.

Figure 8
Effects of piceatannol and pimozone on growth of primary MPN cells
Primary BM cells (MNC) obtained from a patient with JAK2 V617F+ myelofibrosis (PMF) were cultured in control medium (Co) or in medium containing various concentrations of piceatannol (A) or pimozone (B) at 37°C for 48 hours. Then, uptake of ³H-thymidine was measured. Result are expressed as percent of control and represent the mean±S.D. of triplicates.
2.1.5. Characterization of LSC in Acute Lymphoblastic Leukemia (ALL) and Evaluation of the Bone Marrow Stem Cell Niche in Ph+ and Ph- ALL

During the first 3 years of our LB-CO cluster, the lymphoid project-line focused on the characterization of leukemic progenitor cells in chronic lymphocytic leukemia (CLL). These projects were finalized in 2010. In 2011, we focused on ALL, the second major variant of lymphoid leukemias. In a first step we established the phenotype and target expression profile of putative LSC in Ph+ and Ph- ALL. At least in Ph+ ALL, the leukemia-initiating cells are considered to reside within a CD34+/CD38- fraction of the clone. We examined the expression of various markers and targets in CD34+/CD38- or CD123+ BM cells in patients with Ph+ ALL and Ph- ALL, and compared expression profiles with the phenotype of LSC in Ph+ CML and with the phenotype of normal BM SC. Surface expression of target antigens was analyzed by multicolor flow cytometry, and mRNA expression levels in purified CD34+/CD38- and CD34+/CD38+ stem- and progenitor cells by qPCR. As assessed by flow cytometry, CD34+/CD38-/CD123+ cells were found to co-express CD19, CD44, CD52, CD133, CD135, and CXCR4 in all ALL patients examined. In most ALL patients tested (9/12), LSC also expressed CD33. As outlined in 2.1.3., CML LSC and normal SC express a similar phenotype. However, CD19 was only detectable on LSC in ALL patients, and the levels of CD33 and CD52 were higher on ALL LSC compared to normal SC. The IL-1RAP was found to be expressed on LSC in Ph+ ALL, but not on LSC in Ph- ALL or in normal stem cells. By contrast, the SCF receptor KIT (CD117) was detectable on LSC in Ph+ CML and in Ph-ALL, but was hardly detected on LSC in Ph+ ALL. The IL-2RA (CD25) and the SDF-1-degrading surface enzyme DPPIV (CD26) were found to be expression on LSC in a subset of patients with Ph+ ALL, namely those in whom BCR/ABL-p210 was detectable, whereas in patients with Ph+ ALL with BCR/ABL-p190 as well in Ph- ALL or normal marrow, CD34+/CD38- cells did not co-express CD25 or CD26 (Figure 9).

![CD34+/CD38- BM Cells](image)

**Figure 9**
Evaluation of expression of IL-2RA (CD25) and DPPIV (CD26) on LSC in three ALL patients. Expression of CD25 (upper panels) and CD26 (lower panels) on CD34+/CD38- BM LSC was examined by multicolor flow cytometry. Whereas in the patient with ALL with BCR/ABLp210, LSC expressed CD25 and CD26, these targets were not detectable on LSC in the other 2 patients.
The target receptor CD20 was not detectable on LSC, neither in patients with Ph+ ALL nor in patients with Ph- ALL. In most instances, surface expression of target antigens could be confirmed by qPCR. Together, our data show that LSC in Ph+ ALL and Ph- ALL express a unique phenotype including major surface targets. In Ph+ ALL with BCR/ABL-p210, the phenotype of ALL LSC closely resemble the phenotype of LSC of Ph+ CML, confirming the close relationship and similar pathogenesis of these two leukemic conditions. A summary of surface markers and targets identified on LSC in patients with Ph+ ALL and Ph- ALL by flow cytometry is shown in Table 2.

**Table 2**

| Expression of Cell Surface Target Antigens on CD34+/CD38- cells in CML and ALL |
|---------------------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Marker | Ph+ CML (n=30) | Ph+ ALL (n=64) | Ph- ALL (n=5) |
| CD19   | n.t. | + | + |
| CD20   | n.t. | - | - |
| CD25   | +| +/- | - |
| CD26   | +| +/- | - |
| CD33   | +| +/- | +/- |
| CD44   | +| + | + |
| CD52   | +| + | + |
| CD117  | +| - | +/- |
| CD123  | +| + | + |
| CD133  | +| + | +/- |
| CD135  | +| + | + |
| IL-1RAP | +/- | + | - |
| CXCR4  | +| + | n.t. |
| CD221  | +/- | n.t. | - |

*CD33 was expressed on ALL LSC in 9/12 donors tested

In Table 2, phenotypic properties of CD34+/CD38- LSC in Ph+ ALL and Ph- ALL are shown. Expression of surface antigens was determined by monoclonal antibodies and flow cytometry.

In a next step, we examined the endosteal stem cell niche and the vascular stem cell niche in the BM of patients with Ph+ ALL and Ph- ALL. In these investigations, endosteal lining cell were found to stain positive for CD51 (positive control) and CD26, but did not stain positive for CD34 or SDF-1. By contrast, vascular endothelial cells in larger and smaller peri-endosteal vessels, were found to express CD34 and SDF-1 as well as CD26. In both types of cells, VEGF was detectable. No phenotypic differences were found when comparing the phenotype of endosteal or endothelial cells in the BM of patients with ALL with that in the normal BM. These data suggest that endothelial cells and endosteal cells express a unique profile of niche-related antigens, and that both niches act closely together to trigger SC homing and SC properties in ALL and in the normal BM.
2.1.6. Role of Hypoxia, HO-1 Expression, and ROS on ALL Stem Cells

During the first 3 years of our LB-CO project, we were able to show that HO-1, also known as Heat Shock Protein-32 (Hps-32), is expressed in leukemic cells in Ph+ ALL and Ph- ALL. In addition, we were able to show that HO-1 serves as a potential drug target in ALL, as the HO-1-targeting drugs SMA-ZnP and PEG-ZnP induced growth inhibition and apoptosis in these cells. In the 4th year of our LB-CO project, we have extended these investigations, and found that primary CD34+/CD38- ALL LSC express HO-1 mRNA in Ph+ ALL and Ph- ALL (Figure 10). We were also able to show that expression of HO-1 in leukemic progenitors in ALL is dependent on BCR/ABL, as incubation with imatinib resulted in a decreased expression of HO-1 mRNA (Figure 10).

![Figure 10](image)

**Figure 10**
Expression of HO-1 mRNA in ALL progenitor cells
A: CD34+/CD38- and CD34+/CD38+ cells obtained from the BM of a patient with Ph+ ALL were highly purified (>98% purity) from mononuclear cells (MNC) by cell sorting. Purified ALL LSC were examined for expression of HO-1 mRNA by qPCR. Abl was employed as a reference gene. B: Expression of HO-1 mRNA in leukemic progenitors after incubation of cells with control medium (CO) or medium containing imatinib (1 μM) for 48 hours.

We next examined the effects of hypoxia on expression of HO-1 mRNA levels and ROS levels in primary leukemic cells and various cell lines. However, in all cell lines tested, including ALL cell lines, CML cell lines, and MCL cell lines, no effects of hypoxia could be substantiated. We were also unable to demonstrate a major effect of hypoxia on DNA double strand breaks in ALL cells, whereas both camptothecin (positive control) and imatinib produced DNA strand breaks in leukemic cells.

Next, we analyzed expression of other potential targets in ALL cells. Among these, we identified several members of the Bcl-2 family, including Mcl-1, Bcl-xL, and Bcl-2. Unexpectedly, Mcl-1 was among the most prominent anti-apoptotic death regulators expressed in primary ALL cells, ALL LSC, and ALL cell lines. We therefore examined the effects of the pan-Bcl-2 blocker obatoclax on growth and survival of ALL cells. Indeed, obatoclax produced dose-dependent inhibition of proliferation and survival in all ALL cell lines tested and in all primary ALL cell samples analyzed, with reasonable IC50 values (0.01-0.5 μM). Currently, we examine the effects of obatoclax in combination with TKI and other targeted drugs on growth and survival of ALL cells.
2.1.7. Phenotypic Characterization of Neoplastic Stem Cells in Advanced Mast Cell Neoplasms

A number of previous and more recent data suggest that neoplastic progenitor cells in patients with systemic mastocytosis (SM) reside within a CD34+ fraction of the malignant clone. Other studies have shown that like in AML or CML, CD34+/CD38- cells in advanced SM co-express high levels of CD123 and KIT. In the current project, we therefore started to examine the expression of various surface molecules on CD34+/CD38-/CD123 hi stem cells and CD34+/CD38+ progenitor cells in the bone marrow of patients with indolent SM (ISM) and advanced SM, i.e. aggressive systemic mastocytosis (ASM) or mast cell leukemia (MCL). In these analyses, we focused on i) key surface target antigens, ii) surface antigens aberrantly expressed on mast cells in ASM or MCL (but not in ISM), and iii) major stem cell markers. A summary of results obtained by multi-color flow cytometry is shown in Table 3.

Table 3
Phenotype of bone marrow mast cells and bone marrow stem- and progenitor cells in patients with indolent systemic mastocytosis (ISM) and aggressive SM (ASM)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Phenotype in ISM</th>
<th>Phenotype in ASM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mast Cells</td>
<td>CD34+/CD38+</td>
</tr>
<tr>
<td>CD2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD25</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CD26</td>
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<td>CD30</td>
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<td>CD43</td>
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<td>CD52</td>
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<td>+/-</td>
</tr>
<tr>
<td>CD90</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>CD117</td>
<td>+</td>
<td>n.t.</td>
</tr>
<tr>
<td>CD123</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CXCR4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ROBO4</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n.t., not tested; ROBO4, Roundabout 4 (novel SC marker identified in the LB-CO)

Confirming previous observations, CD34+/CD38- stem cells in SM were found to express leukosialin (CD43), KIT, and the “invasion receptor” Pgp-1 (CD44). With regard to key surface target antigens, we found that although both the Ki-1 antigen (CD30) and the Campath-1 antigen CD52 are expressed on neoplastic mast cells in advanced SM, only
CD52 is expressed on CD34+/CD38- stem cells in these patients, which was an unexpected result, that may have clinical implications (as clinical trial-protocols are currently being prepared). Another key finding in our studies was that the IL-3RA (CD123) is expressed on neoplastic mast cells in advanced SM, but not in ISM, an observation that could be confirmed in a series of patients by flow cytometry as well as by immunohistochemistry. Moreover, we were able to confirm that CD123 is expressed at high levels on CD34+/CD38- stem cells in all patients with ISM and ASM. These observations suggest, that CD123, and more importantly CD52, are novel promising targets of therapy in advanced SM. CD30 may also be an interesting target structure, but is not expressed on the more immature progenitor cells in advanced SM.

Next, we screened for novel potential stem cell markers in our recently established cell lines MCPV and VIPA (see below: 2.1.8.) and HMC-1 cells. In these analyses, we compared gene chip and phenotypic results obtained with various stem cell fractions and leukemias and asked for defined subpopulations of cells that would specifically express these antigens. Of all markers examined, two most interesting ‘hits’ were extracted in VIPA cells, namely CD56 and CD90. The CD90 antigen is only expressed in a small subfraction of untransfected VIPA cells, but is expressed on a substantial sub-fraction of KIT D816V-transfected VIPA cells that grow independent of SCF. Currently, we examine whether expression of CD90 is associated with the in vitro growth capability of VIPA cells and with the in vivo oncogenic potential of these cells in NSG mice.

Another key observation was that HMC-1.2 cells consist of two defined subfractions, a CD2+ and a CD2- fraction. In indolent mast cell disease, mast cells express several different adhesion molecules including CD2 and its counter-ligand CD58, and form focal tissue-aggregates, whereas in advanced mastocytosis and MCL, mast cells often lack CD2 and produce a more diffuse infiltration. To explore the functional role of CD2 in the pathology and progression of indolent SM to MCL, CD2+ and CD2− subclones of HMC-1.2 were generated and injected intraperitoneally into pfp/rag2 mice. CD2+ HMC-1 cells formed solid mastocytomas in the peritoneum and lungs, whereas CD2− cells produced a diffuse infiltration in these organs. CD2+ and CD2− HMC-1 subclones all displayed the driver-mutation KIT D816V, exhibited the same growth-kinetics in vitro, and displayed a similar profile of adhesion receptors including CD44 and selectin-ligands by gene chip profiling and flow cytometry. To explore the mechanism of organ invasion, E- and P-selectin-deficient SCID-select mice were employed. While massive HMC-1 infiltrates were detected in the organs of control mice, infiltration was markedly reduced or absent in SCID-select mice. The ‘invasion-receptor’ CD44 was detectable in mast cell infiltrates, with most abundant expression at the invasion-front. Together, these data suggest that selectins and their receptors mediate organ invasion by mast cell progenitor cells in mice, and that CD2-CD58 interactions contribute to focal infiltration in indolent disease. Our data also suggest that the loss of CD2 on mast cell progenitor cells may be an important event during progression from indolent mast cell disease into mast cell leukemia.
2.1.8. Characterization of Novel Mast Cell Leukemia Cell Lines

One of the big issues in mast cell leukemia research is the lack of useful cell lines reflecting stages of mast cell development and molecular signatures related to various forms of mast cell disease, including indolent systemic mastocytosis (ISM), aggressive systemic mastocytosis (ASM), and mast cell leukemia (MCL). In most patients and all categories of the disease (mentioned above), the driver mutation KIT D816V is detectable. However, this observation already suggests that KIT D816V is not the only critical pro-oncogenic molecule (defect) expressed in neoplastic mast cells, and that in advanced mastocytosis, especially in ASM and MCL, additional pro-oncogenic lesions and molecules play an important role, which is also supported by the fact that in advanced SM, KIT D816V-negative subclones may become predominant. Based on results published by us and others, key candidate genes that may drive SM in advanced stages, are mutant forms of IgER, RAS, and TET2, and (other unknown) lesions that lead to activation of LYN and BTK. Other KIT-independent target molecules expressed in neoplastic mast cells in advanced mast cell disease are CD30, CD52, CD123, and CD203c. Some of these target molecules, such as CD30 and CD52, are rather specifically expressed on neoplastic mast cells in ASM and MCL, but not on mast cells in ISM.

During the past few years, we have established a number of novel human mast cell lines, as well as one novel canine mastocytoma cell line. In the current project, we have employed and extended these cell line models with the intention to create a panel of useful lines reflecting various stages of mast cell development and thus various categories of mast cell disease (ISM, ASM, MCL). These cell line models include the MCPV model, an immature SCF-independent mast cell line expressing Large T antigen, hTERT, and RAS G12V, and the SCF-dependent VIPA cell line, a spontaneously transformed immature mast cell line expressing a limited number of chromosome defects. VIPA cells can be further transformed and made SCF-independent by co-transfection of KIT D816V. All MCPV subclones established (n=4) were found to express CD30 and CD52. MCPV cells rapidly engraft NSG mice with mast cell sarcoma and mast cell leukemia. By contrast, VIPA cells express only low amounts of CD30 and CD52. Whereas parental VIPA cells do not engraft NSG mice, KIT D816V-transformed VIPA cells produce a slowly expanding mast cell disease in NSG mice. A highlighting observation was that VIPA cells express a functional IgE receptor on their surface. This is of particular interest, as i) IgE receptor mutants have recently been implicated in mast cell disease progression, and ii) patients with mastocytosis often suffer from mediator-related symptoms after IgE-receptor cross-linking on mast cells. Therefore, our model can be used to examine mechanisms underlying mast cell disease transformation, mechanisms contributing to mediator release in neoplastic (KIT-transformed) mast cells, and mechanisms that contribute to both events. In this regard it should also be mentioned, that VIPA cells and NI-1 cells (a newly generated canine mast cell line) are the first cell line models where KIT mutations and a functional IgE receptor are stably expressed, so that pathogenetic mechanisms relevant to mastocytosis (as described above) can be studied in detail.
2.1.9. Identification and Validation of Molecular Targets in Neoplastic Progenitors in Advanced Mastocytosis and Mast Cell Leukemia

In the 4th project year we focussed on four major targets, namely members of the Bcl-2 family, KIT, Aurora Kinases, and Campath-1 (CD52). In each case, we were able to show that immature mast cell (MC) leukemia lines and CD34+/CD38- cells in patients with ASM and MCL express these antigens. We also screened for expression of other potential target antigens. However, other molecular targets, such as CD30, were not detectable in CD34+/CD38- stem cells in ASM or MCL (see 2.1.7.).

The pan-Bcl-2 family blocker obatoclax was found to inhibit proliferation in primary human neoplastic MC (IC_{50}: 0.01-0.5 μM), in HMC-1.2 cells expressing KIT D816V (IC_{50}: 0.1-1.0 μM), and in HMC-1.1 cells lacking KIT D816V (IC_{50}: 0.01-0.1 μM). The growth-inhibitory effects of obatoclax in HMC-1 cells were accompanied by an increased expression of Puma-, Noxa-, and Bim mRNA, as well as by apoptosis as evidenced by microscopy, Tunel assay, and caspase-cleavage. Retroviral-mediated overexpression of Mcl-1, Bcl-xL, or Bcl-2 in HMC-1 cells, was found to introduce partial resistance against apoptosis-inducing effects of obatoclax (Figure 11). We were also able to show that obatoclax cooperates with several other anti-neoplastic drugs, including dasatinib, midostaurin, and bortezomib, in producing apoptosis and growth arrest in neoplastic MC.

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Figure 11**
Effects of obatoclax on HMC-1.2 cells overexpressing Mcl-1, Bcl-xL, or Bcl-2
Native HMC-1.2 cells and HMC-1.2 cells enforced to express Mcl-1, Bcl-xL, or Bcl-2 by retroviral transduction, were incubated with control medium (Co) or various concentrations of obatoclax for 24 hours at 37°C. A: Apoptotic cells were quantified by light microscopy. Results represent the mean±S.D. of three independent experiments. Asterisk indicates p<0.05. B: Expression of cleaved caspase 3 was detected by Western blotting. Beta actin served as loading control. C: Enforced expression of Mcl-1, Bcl-xL, or Bcl-2 in HMC-1.2 transfected-subclones was confirmed by Western blotting.
We next examined the effects of obatoclax on neoplastic mast cells in various forms of human mastocytosis. In these experiments, we were able to show that obatoclax inhibits the growth and survival of neoplastic MC in all variants, including indolent systemic mastocytosis (ISM), smouldering systemic mastocytosis (SSM), and aggressive systemic mastocytosis (ASM) (Figure 12).

![Figure 12](image)

**Figure 12**
Effects of obatoclax on proliferation of primary neoplastic mast cells
Primary human neoplastic mast cells, isolated from patients with ISM, SSM, and ASM, were incubated in control medium (Co) or in medium containing various concentrations of obatoclax (as indicated) at 37°C and 5% CO₂ for 48 hours. After incubation, 1 μCi ³H-thymidine was added. After 16 hours, ³H-thymidine uptake was measured. Results are expressed as percent of control and represent the mean±SD of triplicates in each donor.

Both HMC-1 subclones were found to express Aurora-Kinase-A and Aurora-Kinase-B at the mRNA- and protein level. Several different Aurora-Kinase blockers tested were found to inhibit the growth and survival of neoplastic mast cells in our experiments. The most potent effects were seen with AS-X (Figure 13), a multikinase inhibitor that blocks the kinase activity of KIT, BTK, Aurora-Kinase-A, Aurora-Kinase-B, ABL, AKT, and FLT3. In addition, we found that this Aurora-Kinase blocker, but not the other Kinase blockers tested, induces a rapid (within 1 minute) dephosphorylation of STAT5 in HMC-1 cells (Figure 13).

![Figure 13](image)

**Figure 13**
Effects of the Aurora-Kinase blocker AS-X on KIT D816V+ HMC-1.2 cells
A: Major dose-dependent effect of AS-X (48 hours) on ³H-thymidine uptake in HMC-1.2 cells. Results are expressed as percent of medium control and represent the mean±S.D. of 3 independent experiments. B: Effects of short-term exposure of HMC-1 cells to AS-X on expression of phosphorylated (pSTAT5) and total STAT5 as assessed by Western blotting.
These data suggest that AX-S is either interacting directly with STAT5 in HMC-1 cells, or is rapidly blocking all STAT5-trIGGERing pro-oncogenic kinases in HMC-1 cells. In consecutive experiments, AS-X induced a rapid G2/M cell cycle arrest as well as apoptosis in HMC-1 cells. AS-X also produced growth inhibition and apoptosis in primary neoplastic mast cells in all patients tested, including ISM, SSM, and ASM. Finally, AS-X was found to cooperate with PKC412 and dasatinib in producing apoptosis in HMC-1.1 cells and HMC-1.2 cells.

With regard to KIT, we confirmed that CD34+/CD38-/CD123+ stem cells in patients with ASM and MCL express this molecular target. The most promising KIT D816V-blocker in ASM and MLC is PKC412 (midostaurin). However, although mediator-related symptoms and organ damage often improve in ASM/MCL patients treated with midostaurin, no long-lasting hematologic remissions are obtained. We examined the in vitro effects of PKC412 and its major metabolites, CGP52421 and CGP62221, in this LB-CO project. All 3 compounds inhibited IgE-dependent histamine secretion with reasonable IC_{50} values (0.01-1 μM). PKC412 and CGP62221 also produced growth-inhibition and de-phosphorylation of KIT D816V in HMC-1.2 cells, whereas CGP52421 showed no substantial effects (Figure 14). Chemical proteomics profiling and drug-competition experiments revealed that PKC412 interacts with KIT and several downstream kinase-targets, and that some of these targets, like Aurora kinase A, are recognized by PKC412 and CGP62221, but not by CGP52421, whereas major IgE-R-downstream targets were all recognized by both metabolites. These data show that the PKC412 metabolite CGP52421 inhibits IgE-mediated histamine release, but does not block proliferation of neoplastic MC, presumably because of altered target-binding capacity. This observation has clinical implications and may explain why mediator-related symptoms improve in PKC412-treated MCL patients even when no hematologic response is obtained.

![Figure 14](image)

Effects of midostaurin (PKC412) and its metabolites on ³H-thymidine uptake in HMC-1 cells. HMC-1.1 cells (left panel) and HMC-1.2 cells (right panel) were incubated with control medium (Co) or various concentrations of PKC412, CGP52421, or CGP62221 as indicated (37°C) for 48 hours. After incubation, uptake of ³H-thymidine was measured. Results are expressed as percent of control and represent the mean±S.D. from 3 independent experiments.
2.1.10. Final Results obtained in Projects on Solid Tumors

Based on the evaluation report, projects on solid tumor cells were finalized in the year 2011. The following key observations have been made and will be followed in other (non-LB Society-funded) forthcoming projects: The CD133+ fraction in the colon cancer cell line HCT116 has a growth advantage over CD133- cells. As assessed by gene chip analysis, the SDF-1-degrading enzyme DPP4 (CD26) was found to be co-expressed in CD133+ HCT116 cells. This is of particular interest as CD26 has recently been described as a prognostic marker in patients with colon cancer. Notably, CD26 levels correlate with disease progression and metastasis-formation in colon cancer patients. In our project, we applied siRNA and shRNA in order to learn whether expression of CD133 or CD26 play a role in tumor growth or survival. However, even a complete iRNA-induced knockdown did not result in growth inhibition or decreased cell survival. Next, we started to explore the effect of the knock-down in a SCID mouse model. Preliminary data suggest that the CD133 knock-down does not block metastasis formation in colon cancer cell lines or other solid tumor cell lines. The ‘CD26 knock down experiments’ will be performed in other non-LB Society-funded projects. In addition, we will try to correlate soluble CD26 levels and CD26 expressed in histologic tumor lesions with the numbers of circulating (metastasis-forming) tumor cells and actual metastasis formation in patients with colon cancer in collaboration with our clinical oncology partners. Our in vitro data obtained with CD133 and CD26 have been submitted for publication.

2.1.11. Final Results obtained in Projects on Melanomas

Based on the evaluation report, projects on melanoma-initiating cells and melanoma-targeting drugs were finalized in the year 2011. The concluding result in this project line is that both the EPOR+ and EPOR- and both the NGFR+ and NGFR- melanoma cells have melanoma-propagating potential in NSG mice. During the 4th project year, we confirmed that melanoma cells indeed express the EPOR by applying fluorochrome-labeled EPO and by measuring EPOR transcript levels in sorted melanoma cells by qPCR. In addition, we examined the effects of recombinant EPO and NGF on growth and survival of melanoma cells. However, both cytokines failed to promote proliferation or survival in primary, EPOR+, melanoma cells, both as single agent or when used in combination. One paper summarizing immunophenotypic results obtained with melanoma cells and melanoma cell subfractions (EPOR+/EPOR-) has been submitted.

Of all targeted drugs tested (n=23), the pan Bcl-2 blocker obatoclax, the Aurora/Multi-Kinase blocker Tozasertib, and the chaperon-targeting drug geldanamycin were found to be the most potent agents acting on melanoma cell growth and survival. All three drugs were found to inhibit the growth and survival of primary melanoma cells and melanoma cell lines at pharmacologically meaningful concentrations. A paper summarizing the effects of various drugs on melanoma cell growth and survival is currently prepared.
2.1.11. Other Key findings and Key Results in 2011

a. Identification of BRD4 as a novel Drug Target in AML LSC

Recent data suggest that BRD4 is a novel drug target in human AML cells. The LB-CO has established this new concept together with collaboration partners at the IMP in Vienna and several institutes in the US, and published these results in the Nature journal in 2011. In a consecutive study, we asked whether inhibition of BRD4 by a small-molecule inhibitor, JQ1, can block growth and survival of CD34+/CD38− and CD34+/CD38+ AML stem- and progenitor cells. Primary AML samples were obtained from 30 patients with freshly diagnosed or relapsed/refractory AML. In unfractionated leukemic cells, submicromolar concentrations of JQ1 were found to induce major growth-inhibitory effects (IC50 0.05-0.5 μM) in most samples, including cells derived from patients with relapsed or refractory AML. In addition, JQ1 was found to induce apoptosis in CD34+/CD38+ stem/progenitor cells as well as in CD34+/CD38− AML stem/progenitor cells in all donors examined as evidenced by combined surface/Annexin-V staining (Figure 15). Moreover, we were able to show that JQ1 synergizes with Ara-C in inducing growth inhibition in AML cell lines. Finally we confirmed expression of BRD4 in highly enriched AML LSC by qPCR. Together, small-molecule inhibition of BRD4 exerts major anti-leukemic effects in a broad range of human AML subtypes, including relapsed and refractory AML. In addition, JQ1 is capable of targeting AML stem- and progenitor cells. Whether BRD4 inhibition is effective in vivo in AML patients remains to be determined. This paper was presented at ASH and selected as best of ASH in 2011.

![Figure 15](image)

Effects of JQ1 on survival/apoptosis in AML stem- and progenitor cells
Left panel: Primary AML cells (n=9) were cultured in control medium (Co) or JQ1 for 48 hours. CD34+/CD38+ AML cells (progenitors/blasts) and CD34+/CD38− AML cells (immature AML stem cells) were examined for signs of apoptosis by combined staining for surface antigens and Annexin-V-FITC. Apoptosis was expressed in percent AnnexinV-positive cells after gating for DAPI negative cells (early apoptotic cells). Results show the percentage of AnnexinV+ cells and represent the mean±S.D. from 9 donors. The right panel shows an example of AnnexinV expression in CD34+/CD38− stem cells in a patient with AML (subtype FAB M4).
b. Identification of Siglec-3 (CD33) as a novel target on CML stem cells

During the past project phase, we found that in almost all patients with AML and CML, CD34+/CD38- and CD34+/CD38+ cells co-express the target receptor CD33. We have extended these analyses in the 4th project year and found that the CD33-targeting drug gemtuzumab-ozogamicin (GO=mylotarg) induces apoptosis and growth-arrest in the CD34+/CD38- and CD34+/CD38+ progenitor cells in virtually all patients with AML and CML. As expected the response to GO was found to correlate with expression of CD33. In fact, in those patients in whom CD33 was clearly expressed on leukemic cells, GO produced a strong effect, whereas in patients, in whom CD33 expression levels were low, the effects of GO were clearly less pronounced. In the 4th project year, we also extended our studies to colony-forming and long term culture-initiating cell assays in CML. In these experiments, preincubation with GO (5 μg/mL) for 2 hours resulted in inhibition of growth of BCR/ABL+ CML CFU-GM and CML BFU-E in all 4 donors tested (Figure 16). The effect of GO on colony-formation was dose-dependent (Figure 16). The presence of BCR/ABL mRNA in individual colonies was confirmed by qPCR.

Figure 16

Effects of GO on colony formation and LTCIC function of CML cells

A: MNC from 4 CML donors were preincubated in control medium or GO (5 μg/ml) for 2 hours. Thereafter, cells were cultured in methylcellulose in the presence of cytokines for 14 days, and the numbers of CFU-GM and BFU-E were counted. Results show colony numbers on day 14 (mean±S.D. from 4 donors). Asterisk: p<0.05 compared to control. B: Dose-dependent effect of GO (2 hour-preincubation prior to seeding) on colony-formation of CML cells in one donor. Results show the numbers of total CFU and are expressed as mean±S.D. from triplicates. C: Numbers of CFU-GM in a long term LTCIC assay. MNC of 3 donors were preincubated in control medium or GO (5 μg/ml) for 2 hours, washed, and transferred to a feeder layer of irradiated M2-10B4 cells for 3 weeks. Thereafter, cells were recovered and CFU growth was determined. Data are the mean±S.D. from 3 donors. Asterisk: p<0.05 compared to control.
In an LTCIC assay, preincubation of CML MNC with GO (5 µg/mL) for 2 hours resulted in a significant decrease of the numbers of CFU-GM in the LTCIC (Figure 16). In 2 donors, MNC were cultured on M2-10B4 cells in the continuous presence of GO (10 or 100 ng/ml). Again, GO was found to reduce the numbers of colony-forming CFU-GM. The presence of BCR/ABL in LTCIC-derived colonies was confirmed by qPCR. Together, these data suggest that GO inhibits the growth and survival of immature CML progenitor cells and CML LSC in vitro. The data have been published in Haematologica. Currently, we extend GO-exposure experiments to our NSG mouse model.

c. Identification of Novel TKI Targets in Neoplastic Mast Cells
As mentioned in 2.1.8., systemic mastocytosis (SM) either presents as a malignant neoplasm with short survival or as an indolent disease with normal life-expectancy and in both conditions, neoplastic mast cells (MC) harbor D816V-mutated KIT, suggesting that additional oncogenic mechanisms are involved in malignant transformation. We found that LYN and BTK are phosphorylated in a KIT-independent manner in neoplastic mast cells in advanced SM and in the mast cell leukemia cell line HMC-1. LYN- and BTK activation was detected in both the KIT D816V-positive HMC-1.2 cell line, and also in the KIT D816V-negative HMC-1.1 subclone. Moreover, KIT D816V did not induce LYN or BTK activation in Ba/F3 cells, and deactivation of KIT D816V by midostaurin did not suppress LYN or BTK activation. siRNAs against BTK and LYN were found to block survival in neoplastic mast cells and to cooperate with midostaurin in producing growth inhibition. Growth-inhibitory effects were also obtained with two targeted drugs, dasatinib which was found to bind to and to block KIT- , LYN-, and BTK activation in neoplastic mast cells by chemical proteomic profiling and MS, and bosutinib, a drug that is known to deactivates LYN and BTK, but does not block the constitutive activation of KIT or KIT D816V. Together, these data suggest that KIT-independent signalling via LYN and BTK contributes to growth of neoplastic mast cells in advanced SM and MCL. Dasatinib and bosutinib disrupt LYN and BTK-driven oncogenic signalling in neoplastic mast cells, which may have clinical implications and explain the synergistic drug interactions seen with PKC412 and Dasatinib. The data have been published in BLOOD.

d. Identification of the PI3 Kinase and mTOR as major Drug Targets in Mast Cells
PI3-kinase and mTOR are two major signaling molecules involved in growth and activation of neoplastic mast cells. We examined the effects of the novel dual PI3-kinase/mTOR blocker NVP-BEZ235 on growth of function of mast cells. NVP-BEZ235 produced strong growth-inhibitory effects on HMC-1 cells, with similar IC50 values in the HMC-1.1 subclone lacking KIT D816V (0.025 µM) and the HMC-1.2 subclone expressing KIT D816V (0.005 µM). Moreover, NVP-BEZ235 was found to exert strong growth-inhibitory effects on HMC-1 cells in a xenotransplant-mouse model. NVP-BEZ235 also exerted inhibitory effects on cytokine-dependent differentiation of normal mast cells, but did not induce growth inhibition or apoptosis in mature mast cells or normal bone marrow cells. The data have been published in PloS One.
Strategic Aims reached in the first 4 years of the LB-CO Cluster:

Several strategic aims formulated for the Cluster were reached within the first 4 years. Probably the most important aim was to establish a robust xeno-transplantation model for various human neoplasms. In fact, the models used in previous studies (also in our labs), i.e. the NOD/SCID model, was found to have several limitations, and in many neoplasms and leukemias, a reproducible repopulation could not be demonstrated. We and others have shown that NSG mice provide a better engraftment model to study tumor- and leukemia-initiating cells. In particular, when using these mice, reproducible engraftment of several myeloid leukemias and many solid tumors can be achieved. For the next period, we will try to further improve myeloid leukemia models by employing NSG<sub>SCFm</sub> mice.

Another strategic aim was to establish phenotypes indicating stem cell-enriched cell populations in various neoplasms and to purify these cells to homogeneity. In myeloid leukemias, a flow-chip-flow approach revealed several novel and more specific stem cell markers, such as ROBO4. In CLL, we were able to identify a hitherto unrecognized (immature) progenitor cell that co-expresses CD5 and CD34. In solid tumors, most stem cell markers could not be verified. Here, further work has to be done to identify novel robust CSC markers. In melanomas, tumor-initiating cells may reside within a EPOR+/CD217+ fraction. However, also EPOR- and NGFR- fractions of melanoma cells may have melanoma-proagitating capacity. A third aim was to define target expression profiles and to examine the effects of various targeted drugs on tumor and leukemic cells and their progenitors. This goal was reached for most of the leukemia models examined.

In addition, we have started to validate these targets by using RNAi technologies and more or less specific targeted drugs, in leukemias (projects on solid tumors and melanomas were finalized in 2011). Finally, we have established several novel cell line models to study leukemic stem and progenitor cells. For the next period of the LB-CO, the most important strategic aim will be to further validate identified LSC markers and targets in human leukemias, and to further establish and validate LSC-niche interactions in the leukemic bone marrow, and to identify underlying mechanisms and related targets.

2.2. Publications and Reports – Overview

A number of original publications and review articles have been published in the project year 2011. Among these papers are several first- and/or senior authorships in Blood, Haematologica, PloS One, and other top journals. In addition, the LB-CO cluster was able to contribute substantially to publications in high impact journals, such as Nature, Cell, or Blood. It should be pointed out, that in each of these cooperations, the preparation and characterization of primary human leukemic (stem) cells was essential. Another important element in these publications is, that they were driven by internal cooperations, i.e. cooperaitions between members and partners of the LB-CO consortium. Finally, it should be mentioned that the LB-CO cluster was involved in several clinical studies published in 2011. A list of publications generated in 2011 is provided in a separate file.
2.3. Patents

No patents were filed in 2011 in our LB-CO Cluster. However, the cluster is actively souting for potentially ‘patentable’ results.

2.4. Conferences

Members of our consortium attended several national and international conferences in the fields of Hematology, Oncology, and Cancer Stem Cell Research. In addition, members of our consortium, in most instances the coordinator, were involved in the organization of national and international conferences and meetings. A highlighting example is the Year 2011 Working Conference on Cancer Stem Cells in Vienna. Major results, such as CD26 expression on CML LSC or the BRD4 data were presented in oral presentations in this Conference as well as at the ASH meeting in San Diego in December 2011.

2.5. Lectures and Presentations

Members of the LB-CO consortium gave a number of lectures and presentations at national and international conferences and meetings, including numerous invited lectures. A list of all presentations is provided in a separate file (publications).

3. Additional Information

3.1. Scientific Cooperations

Scientific Cooperation within the Cluster LB-CO
During the first 4 years of the project, several new scientific cooperations have been established in the LB scientific environment. These cooperations have been extended in the 4th project year, and have been used to strengthen our projects. We have especially extended our strong ongoing cooperation with our colleagues from the departments of dermatology (mastocytosis and mast cell leukemia), pathology (leukemias, cytogenetics), hematology (leukemias and myeloma), and radiation therapy (leukemia models).

Scientific Cooperation with other Groups in Vienna
During the first 4 project years, the core cluster group has established new important cooperation or intensified pre-existing scientific cooperation with a number of groups working in the field of leukemia research and translational research in Vienna. These cooperations were extended in 2011. Cooperations that should be highlighted are the ongoing cooperation with our colleagues at CeMM (Giulio Superti-Furga), the LBI for Cancer Research (Richard Moriggl), and the Vet.Med.Wien (Michael Willmann & Thomas Rülicke). We have also started a collaboration-project (CD52 on MDS/AML LSC) with the Cluster Translational Oncology (Klaus Geissler).
3.2. Organization of Conferences and Meetings

As mentioned above, members of our consortium were involved in the organization of national and international conferences and meetings, including the Year 2011 Working Conference on Cancer Stem Cells in Vienna (September 2-4, 2011).

3.3. Education and Ph.D. Program

The Ph.D. program of the MedUniWien is coordinated successfully by Brigitte Marian who is a key member of our LB-CO consortium. Members of our LB-CO consortium were also involved in teaching and training of students and postdocs in 2011.

4. Aims for the Next Time-Period

Based on results obtained in the first three years of our LB-CO project (2008-2011) and the evaluation report (reviewers’ suggestions) the LB-CO cluster will focus on neoplastic stem cells in leukemias and pre-leukemic conditions in the next project period (2012-2013). Major disorders that will be analyzed are MDS, MPN, AML, CML, ALL, and Mast Cell Leukemia (MCL). Drug-targets, markers, and targeted drugs will be selected based on our previous data and the published literature. In addition, we will initiate a genome-wide screen for pro-oncogenic lesions in various disease models and follow our subclone-and-latency hypothesis of cancer stem cell development. The long-term goal of the project is to develop new improved (potentially curative) therapies for leukemias and related disorders by applying drugs that recognize and eliminate leukemic stem cells.

5. Publications

The Cluster LB-CO published a series of publications in top and standard peer-review journals. A complete list of publications is provided in a separate file.
Ludwig Boltzmann Cluster Oncology

Publications 2011

Original Manuscripts


Wicklein D, Ramos-Leal N, Salamon J, Tamar M, Herrmann H, Valent P, Schumacher U, Ullrich S. Nilotinib and imatinib are equally effective in reducing growth of human eosinophil...


**Review Articles**


Karlic H, Herrmann H, Cerny-Reiterer S. Neue Stammzell-fokusierte Therapiekonzepte in der Hämatol-Onkologie. Der Internist. 2011, in press. **IF: 0.348**


Valent P, Horny HP, Bochner B, Haferlach T, Reiter A. Controverses in the definition and classification of eosinophilia, the hypereosinophilic syndromes, and eosinophilic leukemias. Semin Hematol. in press. IF: 3.038.

Sperr WR, Valent P. Diagnosis, Progression Patterns, and Prognostication in Mastocytosis. Exp Rev Hematol. 2011, in press. IF: 0.459

Other Publications: Letters, Editorials, Book-Chapters, Meeting Reports


Oral Presentations / Lectures


Valent P. Effects of various TKI on Releasability in Mast Cells and Basophils in CML, Mastocytosis and Other Neoplasms. 9th Congress of the European Competence Network on Mastocytosis (ECNM), Istanbul, Turkey, November 19, 2011 (invited).


Gleixner KV. Effects of PTN on Neoplastic MC. 9th Congress of the European Competence Network on Mastocytosis (ECNM), Istanbul, Turkey, November 19, 2011 (invited).


Abstracts


Gleixner KV. Effects of PTN on Neoplastic MC. 9th Congress of the European Competence Network on Mastocytosis (ECNM), Istanbul, Turkey, November 19, 2011.


