Identifying therapeutic targets by elucidating signaling pathways in pediatric lymphoid leukemias
van der Sligte, Naomi Eline

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Summary, discussion, and future perspectives
SUMMARY

Despite intensive therapy, acute lymphoblastic leukemia (ALL) relapse is still a major cause of cancer-related death in children and the survival of children with lymphoid blast crisis chronic myeloid leukemia (CML-LyBC) is even worse.\textsuperscript{1,2} Further improvements in outcome, achieved by dose optimization and combination therapy, have stagnated due to chemotherapy related toxicity.\textsuperscript{3} Therefore, new treatment options are warranted to further improve the cure rates of these children.

Leukemia is the net result of genetic alterations (including point mutations, rearrangements, deletions, and copy number alterations), epigenetic alterations, and/or microenvironmental abnormalities in hematopoietic stem and progenitor cells.\textsuperscript{4-6} These alterations ultimately result in the deregulation of kinase-mediated signal transduction pathways; intracellular signaling cascades involving protein phosphorylation events catalyzed by active kinases.\textsuperscript{7,8} As the malignant transformation of cells highly depends on these deregulated intracellular signaling pathways, protein kinases are an attractive target for cancer therapy.

The introduction of kinase inhibitors for the treatment of CML has profoundly changed the outcome from a fatal disease to a leukemic subtype with a favorable prognosis.\textsuperscript{9} This marks the beginning of a new era of cancer therapy. Over the past decades, genome profiling has vastly increased our understanding of oncogenesis and revealed genetic alterations currently treated with specific kinase inhibitors, for example, \textit{ALK} translocations and \textit{EGFR} mutations in non-small-cell lung cancer and \textit{BRAF} mutations in melanoma.\textsuperscript{10-12} Unfortunately, intensive genome profiling has not yet resulted in the widespread implementation of kinase inhibitors for the treatment of ALL and CML-LyBC, other than kinase inhibitors targeting BCR-ABL. As changes in signal transduction pathways are the net result of genomic, epigenomic, and environmental alterations, the aim of this thesis is to identify potential druggable targets by generating insights in active signal transduction pathways in pediatric lymphoid leukemias.

The implementation of kinase inhibitors in the clinic for the treatment of pediatric malignancies experiences significant limitations. In \textbf{Chapter 2} we provided a short overview of currently used strategies for the implementation of kinase inhibitors as well as reasons why kinase inhibitors has unfortunately not yet resulted in the widespread use for the treatment of pediatric cancer. These reasons include, problems with pediatric drug testing, the complexity of cancer, and resistance to kinase inhibitors. Lastly, we argue that using a personalized medicine strategy combining kinomics, proteomics, and drug screens can bridge the gap between pediatric cancers and the use of kinase inhibitors. In this approach, after initial tumor characterization, kinome and proteome profiling will be performed on patient samples, as well as subject patient cells to a drug screen including multiple kinase inhibitors (either FDA approved or in the pipelines of pharmaceutical companies) to characterize their patient specific cancer profile. Integrating these results will define rational combination therapies. In order to determine treatment effects
on signaling, kinome and proteome profiles will be re-determined after in vitro treatment with potential combination therapies. Ultimately, data integration will result in a comprehensive network of primary signaling pathways, suitable targets for therapy, and drug induced bypass mechanisms.

Previously, we used kinase activity profiling to identify interesting signaling hubs, as well as new potential druggable targets by generating more insights in signal transduction pathways. In Chapter 3 we have employed this technique to provide novel insight into active signal transduction pathways in pediatric ALL and we identified several signaling proteins relevant for ALL pathogenesis and of potential interest for future targeted therapy approaches. In total, we identified 250 commonly phosphorylated peptides, which could be aligned to well-known signal transduction pathways downstream of the precursor B-cell receptor (pre-BCR) and precursor T-cell receptor alpha (pre-TCRA) e.g. the MAPK pathway and the PI3K/Akt pathway, as well as regulators of the cell cycle including components of the p53 and Rb1 signaling pathways. For 27 peptides, differential phosphorylation between BCP-ALL and T-ALL was observed. Among these, BTEB2_S163, CD2_Y281, p73_Y99, OPN_S148, CDX2_S60, and TrkA_Y496 have been previously found to be associated with ALL. To provide a proof of principle for our approach we selected one lead of the list of commonly activated peptides (HGFR_Y1235, also known as c-Met) in order to test its efficacy as a potential target. Despite high peptide phosphorylation intensities in all primary ALL samples, c-Met protein expression could not be detected in any of the four ALL cell lines examined. Since the peptide derived from HGFR_Y1235 is related to sequences corresponding to other proteins including RON (MST1R), we examined RON protein expression and showed that knockdown of RON decreased leukemic cell growth and survival of ALL cell lines. Altogether, Chapter 3 shows that kinome profiling is an elegant approach to study kinase activity by peptide phosphorylation which allows rapid identification of known and unknown signaling proteins relevant to ALL. Furthermore, our list of commonly activated peptides will guide future research into specific proteins as well as targeting options.

In chapter 3 we showed that phosphorylation of a cyclic (c)-AMP response element binding protein (CREB) derived peptide containing residue serine 133 was among the most strongly phosphorylated peptides in the ALL samples. Moreover, the CREB_S133 peptide was more highly phosphorylated in T-ALL compared to BCP-ALL \( (P = 0.024) \). In Chapter 4 we studied the role of CREB as a potential target for treatment in ALL. CREB phosphorylation and mRNA levels showed that CREB expression was significantly higher in ALL compared to normal bone marrow (NBM, phosphorylation: \( P < 0.0001 \), mRNA: \( P = 0.004 \)). In our small pediatric cohort, we observed that patients with high CREB phosphorylation levels trended towards a lower event-free survival relative to patients with low phosphorylation levels \( (P = 0.073) \). In a larger cohort of mostly young adults, high CREB and CREB_S133 levels were correlated with a lower median overall survival compared to patients with normal or below normal levels.
ShRNA mediated knockdown of CREB in ALL cell lines resulted in dramatically decreased cell growth that appeared to be the net result of changes in cell cycle distribution and the induction of apoptosis. Gene expression profiling was performed to explain the observed phenotypic effects. Consistent with a role for CREB in the regulation of cell proliferation, we observed gene expression changes in known CREB target genes regulating cell cycle, apoptosis, and cell proliferation. Interestingly however, within the list of downregulated genes, enzymes involved in glycolysis (GPI, ALDOC, and PKM2), TCA cycle (DLAT and IDH3A), and serine and glycine biosynthesis (PHGDH, PSAT1, and SHMT2) were among the most prominent. The downregulation of these essential genes likely contributes to the observed phenotypic effects observed in response to CREB knockdown. Similar to CREB knockdown, the CREB inhibitor KG-501 decreased leukemic cell viability and induced apoptosis in ALL cell lines, as well as primary T-ALL samples, with cases showing high CREB phosphorylation levels being more sensitive than those with lower phospho-CREB levels. Together, the in vitro findings of this study support an important role for CREB in the survival of ALL cells and identify this transcription factor as a potential target for treatment in ALL.

Genome wide approaches in ALL have aenlarged the list of risk predictors and included IKZF1 deletions as an unfavorable prognostic factor.\textsuperscript{16-18} IKZF1 deletions can be identified in approximately 70% of the children with Philadelphia chromosome positive (Ph\textsuperscript{+}) ALL and in 10-15% of the children with Philadelphia chromosome negative (Ph\textsuperscript{-}) ALL. Although IKZF1 deletions in children are most commonly found in a Ph\textsuperscript{-} background, the effect of IKZF1 deletions on signaling pathways in Ph\textsuperscript{-} ALL have not been extensively studied. Therefore, in Chapter 5 we aimed to study the effect of IKZF1 deletions on active signal transduction pathways using kinome profiling. Kinase activity profiles of 31 IKZF1 wild type patients and 14 patients harboring an IKZF1 alteration (13 deletions and 1 gain) were generated. Unsupervised hierarchical cluster analysis showed no clustering based upon IKZF1 status, karyotype, or other copy number alterations. Thirty-nine peptides were differentially phosphorylated between IKZF1 deleted and IKZF1 wild type pediatric Ph\textsuperscript{-} BCP-ALL patients. Nevertheless, absolute differences of peptide phosphorylation intensities between IKZF1 deleted and wild type samples were small. To focus more closely on active signal transduction pathways, we determined peptide phosphorylation of proteins involved in important signaling pathways for BCP-ALL cell proliferation and survival (e.g. the BCR signaling pathway, the MAPK, PI3K/Akt/mTOR, JAK/STAT5 signaling pathways), adhesion pathways, and regulators of the cell cycle. Comparing phosphorylation intensities of peptides associated with these main signaling pathways showed no differences in pathway activation between the two groups. In addition to the kinome profiles, we performed western blot analysis of 14 pediatric BCP-ALL samples. These western blot results showed large variations in phosphorylation levels between the different ALL samples, independent of IKZF1 status. Based on these results we conclude that although IKZF1 deletions appear to be an important clinical prognostic factor, we were unable to identify a unique IKZF1 dependent
protein expression signature in pediatric Ph- ALL and consequently no specific targets for future therapy of Ph- IKZF1 deleted BCP-ALL could be identified.

With the introduction of tyrosine kinase inhibitors (TKIs), CML has transformed from a fatal disease to a leukemia subtype with a favorable prognosis.\textsuperscript{9} However, once a blast crisis (BC) has occurred, treatment options are limited in the terms of months.\textsuperscript{2} Therefore, early recognition of patients at risk of developing a BC is desirable. In the \textbf{Chapter 6} we used Multiplex Ligation-dependent Probe Amplification (MLPA) analysis to screen for the presence of copy number alterations (CNAs) in a large cohort of pediatric CML cases in order to investigate whether B-cell lymphoid leukemia specific copy number alterations (CNAs, e.g. \textit{IKZF1}, \textit{PAX5}, and \textit{CDKN2A} deletions) could be detected in pediatric CML-CP and used to predict disease progression to LyBC. Copy number alterations were detectable in material from one of the CML-AP and in all of the CML-LyBC patients, while no CNAs were found in any of the 77 CML-CP samples. Recurrent deletions at time of CML-LyBC were found in \textit{IKZF1}, \textit{PAX5}, and the \textit{CDKN2A/B} locus. Based on this study, we conclude that clonal CNAs are rare or even absent in pediatric CML-CP but are a hallmark of disease progression.

The activation of signaling pathways, independent of BCR-ABL1 activity, might be involved in disease progression to CML-LyBC and CML-LyBC cell survival. Previous studies have described a roll for Akt, STAT3, and FAK activation in the development of imatinib resistance in CML, with a subsequent higher risk of progression to blast crisis.\textsuperscript{19-24} In \textbf{Chapter 7} we have studied the role of Akt, STAT3, and FAK for the survival of CML-LyBC cells to identify new potential therapeutic targets. Phosphorylation of these three targets was highly variable between four CML-LyBC cases, and consistently, differential effects of target inhibition on cell survival were seen. Nevertheless, these preliminary results show that the direct use of kinase inhibitors might be suitable for the treatment of CML-LyBC, guided by kinase activity profiles in individual patients. This further emphasizes the need for personalized approaches to identify patient-specific targets for therapy. Hence, to extend on these preliminary observations, kinome and proteome profiling may be successfully used to identify active signal transduction pathways in CML-LyBC.
GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Kinase activity profiling

Deregulated intracellular kinase-mediated signal transduction pathways are implicated in the genesis and maintenance of cancer either by stimulating growth (e.g. increased cell survival and cell growth) or by contributing to resistance to therapy. Kinases are enzymes catalyzing protein phosphorylation by transferring phosphate groups from adenosine triphosphate (ATP) to specific proteins. Kinases can acquire tumor-promoting capacities by genetic alterations including mutations and translocations that give rise to constitutive active kinases. These constitutive active kinases have proven to be very effective targets for targeted therapy with small molecule inhibitors as shown for BCR-ABL1 positive chronic myeloid leukemia (CML), ALK-rearranged non-small cell lung cancer (NSCLC), and BRAF mutated melanoma. In addition to these cancer subtypes with known “driver” mutations, intracellular kinase-mediated signal transduction pathways are required for malignant transformation in all cancer types. Therefore, kinases have become one of the most intensively targeted class of enzymes for therapy.

In this thesis we aimed to identify therapeutic targets by generating insight into active signal transduction pathways in pediatric lymphoid leukemias using high-throughput kinase activity profiling. Active signal transduction pathways were determined in various ALL subtypes including BCP-ALL and T-ALL (chapter 3) and in IKZF1 wild type versus deleted BCP-ALL (chapter 5). This in vitro array enables kinases in the sample lysate to phosphorylate peptides derived from known phosphorylation sites from human protein sequences. In this thesis kinome profiling generated insight into active signal transduction pathways in ALL including the activation of the well-known signaling pathways MAPK and PI3K/Akt downstream of the pre-BCR and pre-TCRA and regulators of the cell cycle/p53 pathway, as well as the identification of targets previously described in either BCP-ALL or T-ALL. In addition, we created a list of targets of potential interest for future therapy approaches in pediatric ALL. This list includes growth factor receptors, intracellular kinases of different signaling pathways, and transcription factors. The validity of our approach was shown with the identification of RON and CREB as attractive targets for future investigations. Despite these promising results, as with other approaches, we came across several limitations. First of all, kinase activity profiling is an in vitro approach without spatiotemporal regulation mechanisms and the tertiary structure of phosphorylation sites causing the relationship between in vitro data and the in vivo situation not to be 1:1 comparable. Moreover, since we are using peptide sequences of approximately 11 amino acids in length, sensitivity and specificity of each individual target peptide e.g. which individual kinase recognizes a target peptide and how efficiently the kinase phosphorylates that peptide is largely unknown. Additionally, it is difficult to find a peptide substrate specific to one kinase. As we experienced in chapter 3 where we observed phosphorylation of the peptide derived from c-Met despite undetectable membrane and intracellular c-Met protein expression, cross-phosphorylation might occur making validation experiments required to confirm novel kinome data findings.
This issue might be overcome by combining individual peptide phosphorylation events, rather than evaluating individual peptides. Another disadvantage of kinome profiling is that this approach only provides information about end point phosphorylation events. Consequently, this readout gives limited information about slowed down or accelerated reactions, which are frequent processes in treatment responses or oncogenesis, respectively. For the future, the use of peptide arrays may be improved by measuring kinetic phosphorylation rates rather than end point measurements, as already implemented in the Pamgene microarrays. Finally, data interpretation and data processing are still a major challenge to fully explore the wealth of information acquired by kinome profiling. In this thesis we studied peptide phosphorylation instead of upstream kinase activity since peptide sequences are correlated with a variable amount of upstream kinases and the interference of spot intensities into in vivo upstream kinase activity is not straightforward. In addition, we generated an overview of active signal transduction pathways in pediatric acute lymphoblastic leukemia by creating a disease network based on known protein-protein interactions of the commonly activated peptides according to the STRING database and annotated these peptides to known signal transduction pathways using the KEGG and PhosphoSite pathway databases. Using these databases, kinome profiling results are implemented in a context of known biological information. However, by the use of mathematical and computational models for the analyses of network interpretation, kinome profiling might also be able to provide new biological data. This will be an important goal to reach in the near future resulting in a comprehensive use of all the information kinome profiling may provide. In conclusion, peptide arrays are able to provide detailed insights in the dynamics of intracellular signaling pathways and the complexity of cancer cells, as shown in this thesis. When these results can be combined with data from all different omics technologies such as genomics and transcriptomics using inventive bioinformatics programs, the impact of the data will be even stronger.

Receptor tyrosine kinases and transcription factors as putative druggable targets

In our list of commonly activated peptides we noticed several growth factor receptors (chapter 3). Although receptor tyrosine kinase inhibitors (RTKIs) have been implemented in the treatment of specific solid tumors, FDA approved inhibitors targeting various receptor tyrosine kinases e.g. sorafenib, crizotinib, and lapatinib are not yet used for the treatment of leukemias. Although evidence from literature is limited, expression of several receptor tyrosine kinases has been reported in leukemia and targeting these receptors was shown to be effective, alone or combined with conventional therapies, in the treatment of ALL. For example, EGFR2 (ErbB2) is expressed in approximately 30% of the patients with BCP-ALL and in vitro inhibition of EGFR2 reduces cell proliferation especially when combined with BCR-ABL tyrosine kinase inhibitors in Ph+ ALL. Additionally, upregulation of RTK-ligand levels has been frequently observed following
kinase inhibition and is able to activate downstream intracellular signaling pathways to induce therapy resistance.\textsuperscript{36,37} These results, in combination with our kinome profiling results, show that although mutations in receptor tyrosine kinases are rare in leukemias, compounds directed at these targets might be of potential interest in the treatment of leukemia.

In addition to receptor tyrosine kinases and intracellular kinases, several transcription factors including CREB, Bcl-2, c-Myc, and NF-kappaB were found in our list of commonly activated peptides (chapter 3). Transcription factors have traditionally been considered too difficult to target and pharmacological inhibition of transcription factors is usually achieved by the inhibition of upstream phosphokinases or endogeneous ligands.\textsuperscript{38-41} However, targeting upstream phosphokinases lacks specificity because of the multiple downstream effects of one single intracellular kinase and its numerous feedback loops.\textsuperscript{38} Consequently, targeting transcription factors is likely to be a more specific approach, but targeting the interaction between DNA-binding proteins and DNA using inhibitors is technically challenging.\textsuperscript{38,42,43} It has been hypothesized that targeting the protein-protein interactions of transcription factors with their co-activators, as for KG-501 the CREB inhibitor we used in chapter 4, might be a pharmaceutical possibility to target transcription factors.\textsuperscript{38,44} Since pharmacological inhibition of protein-protein interactions are likely to represent one of the next major classes of therapeutic targets, drugs targeting transcription factors are approaching and may be of greater importance in the near future.\textsuperscript{38}

**Targeted therapy: from bench to bedside**

**Preclinical models**

Since the discovery of imatinib for CML, cancer research has been focused on the identification of new therapeutic targets for the treatment of other malignancies. Validation of these targets in cancer cell lines is usually one of the initial steps in drug development. Cell line models are easy to handle and manipulate, show reproducible results, and are an inexpensive model to test a large number of potential drugs before committing to large scale \textit{in vivo} trials.\textsuperscript{45,46} In addition to the use of cell lines for cytotoxic assays to determine drug sensitivity patterns, cell lines can also be used to study drugs’ mechanisms of action and can be used for the research of signaling pathway adaptations in response to anticancer drugs.\textsuperscript{47-49} Although human cancer derived cell lines are the most widely used models to test therapeutic strategies, the limitations of cell line models are well known.\textsuperscript{49,50} For example, since not all cancer types can grow indefinitely \textit{in vitro}, those that do grow have acquired new mutations that allow these cells to adapt to their artificial environment.\textsuperscript{51} Furthermore, cell lines do not recapitulate the molecular heterogeneity of human tumors.\textsuperscript{52} Improving the preclinical evaluation of targeting compounds, focusing on effectivity and toxicity, is necessary and of great importance since only about 5\% of the identified putative anticancer compounds demonstrate sufficient clinical activity in phase III trials.\textsuperscript{52,53} Although animal models are essential to study the effects of newly
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identified drugs, improvements of in vitro models that more closely mimic tumor heterogeneity as well as the in vivo tumor microenvironment will be the first step. This will be challenging since acute lymphoblastic leukemia cells cannot be sufficiently cultured in vitro, despite specific culture medium supplemented with growth factors including cytokines, FLT3-ligand, and stem cell factor. Attempts to improve in vitro models for the culture of primary ALL cells might include, for example, a culture system mimicking the bone marrow niche including optimal oxygen levels and cell-cell and cell-stroma contact, like a hydrogel-based 3D-culture system of mesenchymal stromal cells.

Clinical trial design for targeted therapy

In addition to improvements of preclinical study models, the design of traditional clinical trials also needs to be adapted to kinase inhibitors. Traditionally, phase I trials are performed to identify a maximum tolerated dose and side effects which can be used into phase II trials to determine drug activity. In contrast to conventional cytotoxic drugs, the relationship between target effect and toxicity may not be linear for kinase inhibitors. Kinase inhibitors may have a plateau in effect, meaning a higher dose will not improve clinical efficacy and consequently patient benefit. Additionally, kinase inhibitors do not necessarily induce toxicity in healthy tissues. Therefore, the aim of phase I clinical trials using kinase inhibitors should be to identify the optimal biological dose. This is defined as the dose at which there is maximal inhibition of the target with acceptable toxicity. Safety, side effects, pharmacokinetics, and pharmacodynamics should be documented at that dose. As the primary end point will be target inhibition, enrolling patients with different forms of cancer is feasible. Obviously, as kinase inhibitors target specific kinases, trials should include only patients that express the target. Summarizing, insights in the mechanisms underlying oncogenesis have changed the paradigm of cancer treatment with promising results of kinase inhibitors in the laboratory. In the next years we should further improve the design of preclinical models by optimizing in vitro culture systems and clinical trials by defining new adapted study objectives to successfully introduce these new types of drugs into the clinic.

Challenges in targeted cancer therapy

Despite the success of imatinib for the treatment of CML, the clinical translation of kinase inhibitors for the treatment of patients has been sub-optimal. Drug resistance to kinase inhibitors by cell-intrinsic mechanisms, the cross-talk of cancer cells with their microenvironment, and tumor heterogeneity are major challenges when considering the use of targeted therapy in the context of cancer.
Therapy resistance

As outlined in chapter 2, several mechanisms of drug resistance have been described. First of all, acquired mutations might cause resistance to kinase inhibitors in mainly two ways: (1) mutations altering the target, for example kinase domain mutations, confer resistance by decreasing the efficiency of the inhibitor and (2) new mutations may activate alternative survival pathways and circumvent the inhibitory effect of a given drug.

Secondly, therapy resistance can be mediated by dynamic reprogramming e.g. alternated routes of kinase pathway activation in response to pharmacological disturbance. Alternated activation of kinase-dependent signaling pathways might either be upstream or downstream of the target pathway or by the activation of bypass signaling pathways. Additionally, the microenvironment might activate survival signaling pathways by cell adhesive interactions or by the secretion of soluble growth factors or cytokines. Therapy resistance by dynamic reprogramming may be defeated by simultaneous inhibition of the primary pathway as well as the bypass pathway (combination therapy). Previously, we have shown that a combination of kinome and proteome profiling is able to predict signaling pathway adaptations and can be used to define rational combination therapies. This observation might be valuable for future approaches to improve the clinical success rate of kinase inhibitors with a pivotal role for signaling dynamics. In chapter 2 we argue that using a personalized medicine strategy can improve the implementation of kinase inhibitors. In order to minimize the occurrence of dynamic reprogramming, the patient specific signaling profile will need to be established both at time of diagnosis and after the in vitro treatment of selected combination therapies to determine basal signal transduction activity as well as drug induced signaling. Data integration will ultimately result in a more comprehensive view of active signal transduction pathways during treatment, including potential bypass mechanisms.

Tumor heterogeneity

Inter- and intratumor heterogeneity add an additional level of complexity to the understanding of cancer. Intertumor heterogeneity was documented using genome-wide analysis subdividing acute lymphoblastic leukemia in various subtypes with diverse kinase-activating lesions, making targeted therapy approaches only appropriate for specific subtypes. Evaluating intertumor heterogeneity at the level of drug sensitivity, is even more complex than initially hypothesized based on the various genetic subtypes. As signaling dynamics is the net result of genomic, epigenetic, and microenvironmental abnormalities, drug sensitivity patterns to kinase inhibitors showed heterogeneous results within genetic subgroups. In chapter 5 we performed kinome profiling in IKZF1 deleted BCP-ALL patients to identify potential targets for the treatment of this ALL subtype. Although IKZF1 deletions are an independent clinical prognostic factor, no IKZF1 distinct protein expression profile could be established using kinome profiling and western blot analysis. Suggesting that within predefined ALL subtypes, in chapter 5 patients
harboring IKZF1 deletions, heterogeneous subgroups exist. Similarly, intertumor heterogeneity was seen in lymphoid blast crisis CML, where we observed various drug responses among different CML-LyBC cases (chapter 7). Intertumor heterogeneity challenges the implementation of kinase inhibitors into the clinic since subpopulations suited for specific targeted therapies must be determined accurately and clinical trials based on the “one-size-fits-all” strategy are in general disappointing. Although we have analyzed the kinome profile results within predefined subgroups, results might also be analyzed individually and patients may be classified according to patient specific signaling profiles. This would enable the identification of patients with similar signaling dynamics including specific targets for therapy instead of targets of potential interest for entire ALL subtypes. As we have observed an overlap of active signal transduction pathways within different subtypes of cancer and drug screen approaches showed no complete segregation based upon leukemia types.\(^6\) It might be interesting to perform kinome and proteome analyses in a large cohort of various pediatric malignancies. Such an analysis will be able to determine signaling dynamics heterogeneity, as well as similarities within various malignancies to evaluate signaling specific potential druggable targets instead of disease specific targets based on signaling.

Next to intertumor heterogeneity, intratumor heterogeneity potentially represents the greatest challenge to deliver effective personalized cancer therapy.\(^{25,60,67}\) Intratumor heterogeneity develops in space and time, leading to a complex mix of clones.\(^{25,60}\) Subclonal diversity was also reported in ALL, showing ALL as a heterogeneous disease at diagnosis and varying over time continuously with the development and progression of disease.\(^6\) In the majority of the ALL cases, relapses arise from the emergence of a minor subclone through a genetic evolution following Darwinian logic by different responses to antileukemic therapy.\(^{60,69,70}\) Single-cell sequencing allows a better understanding of intratumor heterogeneity required to clarify the events mediating cancer formation, treatment resistance, and the implementation of clonal specific molecular targets.\(^{71}\) The effects of genetic heterogeneity on the heterogeneity at the protein level are largely unknown. Single cell proteomic approaches such as single-cell western blotting are extremely challenging, but upcoming.\(^{72}\) Nevertheless, it has been hypothesized that heterogeneity at the protein level of cancer cells is less complex than could be expected from genetic heterogeneity.\(^{73,74}\) Especially, since all of the 138 known driver genes can be classified into one or more of 12 co-operating pathways.\(^{75}\) With the current techniques single-cell kinome profiling is not yet available to study the effect of genomic intratumor heterogeneity on the level of kinome profiles. However, by redetermining kinome and proteome profiles after in vitro treatment with kinase inhibitors or combinational treatment, the signaling dynamics of therapy resistant subclones will become more prominent in this second overview; providing the selection of kinase inhibitors able to target both the major subclone as well as the therapy resistant subclones.
Systems biology to guide future personalized medicine

As illustrated by the pitfalls of current targeted therapies, cancer is an extremely complex disease and this complexity remains a major challenge for the implementation of individualized medicines into the clinic. It has been hypothesized that system biology approaches can manage and model this complexity through the integration of cellular signaling networks between cells, stroma, organs, and the entire organism.\textsuperscript{32,67} Cancer systems biology aims to develop effective predictive models for the validations of new therapies and drugs by analyzing how the intracellular networks of normal cells are disturbed during oncogenesis.\textsuperscript{67} For example, mathematical modeling can integrate the evolutionary nature of individual cancer genomes to analyze and predict the dynamics of heterogeneous tumor populations on therapy response.\textsuperscript{67,76} Since cancer is a multi-factorial disease, comprising genetic, transcriptional, epigenetic, and signaling alterations, information from one layer can be used to infer alterations in another layer.\textsuperscript{32} Therefore, cancer systems biology might play a central role in future personalized medicine.

CONCLUSIONS

Despite the great efforts made to identify targets for the treatment of lymphoid leukemias, the clinical implementation of targeted drugs remains a challenge. This thesis describes the use of kinome profiling for the identification of potential therapeutic targets in lymphoid leukemias. Based on the obtained kinome profiles we established a number of putative druggable targets, of which RON and CREB were validated by \textit{in vitro} studies. Although the implementation of targeted therapy remains challenging due to cancer complexity, ongoing developments of high-throughput techniques combined with systems biology should facilitate the selection of therapeutic targets with the ultimate goal to cure every child with lymphoid leukemia.
REFERENCES


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