Investigating possible bypass mechanisms to sensitize AML blasts for combination therapy
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Summary
Discussion
Future Perspectives
Conclusion
Summary

In this thesis, the dynamic adaptation potential of AML blasts was investigated to define their facilitating bypass mechanisms as escape routes that circumvent initial targeting strategies. We found RTK activation as possible initiators of bypass mechanisms in AML. To this extend, we challenged to interfere with the following ligand/receptor axis in AML e.g. VEGFC/VEGFR-2, NGF/TRKA, and EfnB1/EphB1.

The human VEGF family consists of VEGFA, VEGFB, VEGFC, VEGFD, and PIGF (placenta growth factor). The best studied, and probably most important VEGF family members in AML are VEGFA and VEGFC. VEGF ligands can bind to one or more VEGF receptors; VEGFR-1 (i.e. FLT-1 fms-related tyrosine kinase-1), VEGFR-2 (i.e. KDR, kinase insert domain receptor), or VEGFR-3 (i.e. FLT-4 fms-related tyrosine kinase-4) (chapter 2). Proteolytic processing of VEGFC regulates the receptor specificity. VEGFR-2 preferentially binds the fully processed mature form of VEGFC, while VEGFR-3 can bind the non-processed forms as well. VEGFC and the corresponding receptors are overexpressed by AML blast cells. VEGFC induces the phosphorylation of both VEGFR-2 and VEGFR-3 in AML cells. The VEGF signaling promotes autocrine AML blast cell proliferation, survival, and chemotherapy resistance. In addition, VEGF signaling can mediate paracrine vascular endothelial cell controlled angiogenesis in AML. Both effects are presumably explaining the association of high VEGF levels and poor therapeutic outcome. More recently, researchers focusing on bone marrow stem cell niches demonstrate a role for VEGF signaling in the preservation of several cell types within these niches. The bone marrow niches are proposed to be a protective microenvironment for AML cells that could be responsible for relapses in AML patients. This implies the need of sophisticated VEGF targeting agents in AML.

High vascular endothelial growth factor C (VEGFC) expression predicts adverse prognosis in pediatric and adult acute myeloid leukemia (AML). AML patient samples expressed higher levels of VEGFC and VEGFR-2 protein as compared to NBM controls. In chapter 3, we challenged to target VEGFC by using a human VEGFC antibody in CD34+ sorted AML patient samples. Targeting primary CD34+ pediatric AML cells by anti-VEGFC treatment significantly enforced myelocytic differentiation by 3.3-fold, characterized by increasing numbers of CD11b, CD14 or CD15 expressing cells in both short-term
colony forming cell (CFC) assays and long-term cultures-initiating cell (LTC-IC) assays. Anti-VEGFC treatment reduced the short-term and long-term expansion potential of CD34+ AML cells by 30-50%. The presence of identical FLT3-internal tandem duplications (FLT3-ITD) in untreated and anti-VEGFC treated cultures underscored that the increased mature myelocytic population is of leukemic origin. Normal bone marrow (NBM) CD34+ cells showed an extreme latency of anti-VEGFC induced myeloid differentiation potential. Anti-VEGFC treatment functions through inhibition of myeloid proliferative maintenance signals by reduced phosphorylation of MEK1/2 and STAT5a/b. Gene expression array analysis of anti-VEGFC treated AML patient samples revealed a significant induction of glycolytic genes HK3 and PFKL. Anti-VEGFC treatment induced glycolysis was confirmed in THP-1 AML by 2-fold increase in the glycolytic flux without affecting the glucose oxidation, glutaminolysis and oxidative pentose phosphate pathway. The increase in glycolysis was confirmed by increased lactate production. Inhibition of glycolysis by PFKFB3 targeting strategies (pharmacological or by shRNA) and anti-VEGFC simultaneously reduced the expansion potential of THP-1 AML cells as compared to single therapies and controls. Combining glycolysis inhibition (PFKFB3 inhibitor 3PO) and anti-VEGFC reduced the maximum glycolytic capacity and resulted in a complete shutdown of the mitochondrial basal and maximum respiration capacity. Next, we investigated combination therapy efficiency in AML THP-1 xenograft model in NSG (NOD-SCID/IL2γ-/-) mice. Tumor development was significantly decreased in mice that received combinations of 3PO and anti-VEGFC treatment. THP-1 AML xenografts in NSG mice revealed that the combination of 3PO and anti-VEGFC resulted in a better efficiency of glycolysis inhibition in the tumors as compared to control mice injected with either 3PO or anti-VEGFC, suggesting that anti-VEGFC therapy leads to a better intra-tumor delivery of 3PO. So far, these findings indicate an important regulatory function for VEGFC in CD34+ AML cell fate decisions. Anti-VEGFC therapy reduced CD34+ AML blast expansion via Erk inhibition and enforced myelomonocytic differentiation through FOXO3A suppression. Dynamic adaptation was shown by anti-VEGFC induced glycolytic acceleration for lactate production via the PFKFB3/PFKL axis. Inhibition of VEGFC and glycolysis (3PO) shows significant in vivo therapeutic potential, creating opportunities for additional treatment besides high dose chemotherapy.

Deregulated signaling activity is frequently observed in leukemia, leading to induced proliferation, migration, survival, and chemotherapy resistance of leukemic
However, single kinase-targeted cancer therapies can default when cancer cells bypass through alternative routes, facilitating therapeutic resistance. In order to circumvent the constraints given by an inhibitor requires analysis of kinome reprogramming upon mono-treatments to develop the most successful combination therapy approach for disease specific subgroups. Therefore, we explored the intracellular signaling networks and study the dynamic changes resulting in reprogramming of the kinome network, with the goal to define combinational therapeutic strategies. In chapter 4, we succeeded using combined high-throughput approaches for kinomic and proteomic profiling to identify specific aberrant kinase signatures in MLL-rearranged AML as compared to NBM. The altered activated kinase signatures of a comprehensive set of MLL-rearranged AML patient samples resulted in a detailed map of the overall kinase activity and phosphorylation of signal transduction molecules, which allowed the selection of possible druggable targets e.g. MEK, JNK, and CREB. Pharmacological MEK inhibition in primary MLL-rearranged AML demonstrated to be most successful in reducing the MLL-rearranged AML cell survival as compared to other karyotype AMLs, without showing cytotoxicity in NBM, whereas for CREB and JNK inhibitors MLL-rearranged AML cells were equally affected as NBM cells. Dynamic kinome reprogramming of signaling networks in response to MEK therapy did occur, by inducing the activation of VEGFR-2 to bypass the initial MEK inhibitory effects. Combined MEK and VEGFR-2 inhibition showed a stronger reduction in MLL-rearranged AML cell survival in a subgroup of patient samples. This advantageous strategy allows rational design of successful and selective combination therapy for an AML subgroup of high VEGFR-2 expressing MLL-rearranged AML patients.

Currently, many RTK inhibitors have passed clinical phase 1 and phase 2 trials and became available for patient treatment. The first clinical studies using Vatalanib (PTK787/ZK22584), Semaxanib, Sunitinib, Axitinib, Sorafenib, Midostaurin and Cediranib report heterogeneous results in AML patients, which require additional research to address the clinical significance more precisely. Interestingly, we and other groups found that receptor tyrosine kinases are important regulators of therapeutic resistance facilitating pathways in different types of cancers. RTKs in AML have been shown to facilitate cellular resistance mechanisms e.g. FGFR1 and VEGFR-2/KDR. Using the multiple RTK inhibitor PTK787/ZK222584 (PTK/Vatalanib), we previously showed eradication of a large part of CD34+ pediatric AML blasts in vitro. This study
revealed the inhibition of the PI3K/Akt pathway activation as contributor for the observed effects. Nevertheless, a subset of CD34+ AML blasts survived PTK787/ZK222584 inhibition showing only a small delay in blast expansion potential. Therefore, in chapter 5, we aimed to investigate AML therapeutic resistance opportunities upon PTK787 treatment for multi-RTK inhibition in primary pediatric AML samples by performing comprehensive analysis of dynamic kinome reprogramming, which enabled us to study resistance pathways without a priori knowledge. PTK787 treatment of primary pediatric AML samples showed kinase activation of peptides belonging to the TRKA signaling pathway. Subsequent western blot analysis showed sustained TRKA phosphorylation upon PTK787 treatment which upon activation by NGF provided a survival mechanism for AML cells. Using this study design, we defined a novel new therapeutic approach by using PTK787/ZK222584 together with TRKA inhibition as a more efficient strategy than single agents in the eradication of the AML cell survival in cell lines and primary pediatric AML blasts.

RTKs involvement in therapy resistance can be affected by many post-transcriptional modifications, of which we mainly studied protein phosphorylation for protein activation in previous chapters. Nevertheless, also pre-transcriptional modifications can lead to suppression of transcription and translation involved in cancer. Ephrin signaling has been shown to contribute to the pathogenesis of many solid tumors with respect to tumor growth, tumor cell survival, angiogenesis, and metastasizing capacity. Recently, an aberrant DNA methylation status of ephrin receptors and ligands was described to be associated with outcome in acute lymphoblastic leukemia. In acute myeloid leukemia (AML), we found an active EphB1 suppression by promoter hypermethylation (chapter 6). Therefore, we challenged to evaluate the role of EphB1 receptor forward signaling in AML. We investigated the influence of the EphB1 receptor forward signaling in THP-1 (EphB1\textsuperscript{high}), HL60 (EphB1\textsuperscript{int}), and MOLM13 (EphB1\textsuperscript{low}) AML cell lines through exogenous stimulation with the EphB1 ligand; EfnB1. EfnB1 stimulation of the AML cell lines demonstrated to reduce leukemic growth solely in the EphB1\textsuperscript{high} AML cells. Subsequently, EfnB1 stimulation induced apoptosis in the EphB1\textsuperscript{high} cells. These phenotypic effects could be assigned to a G2/M cell cycle arrest via restoration of the DNA damage control system, by activating the ATR/Chk1 axis. Subsequent this was validated by induced p53 DNA binding and subsequent induction of p21 transcription. To verify whether the EfnB1 induced cell cycle arrest is EphB1 specific, we enforced EphB1
expression in HL60 and MOLM13 EphB1\textsuperscript{low} AML cells by constitutive EphB1 overexpression. Again, we found increasing levels of Chk1 and cell cycle inhibition through sustained CDK1\textsuperscript{Tyr15} phosphorylation. By using a previously performed kinase array, we found reduced EphB1 peptide activation in AML samples as compared to NBM controls, while other ephrin receptors were not found to be significantly different. Subsequently, in 20% of the pediatric AML patient bone marrow samples, we found EphB1 hypermethylation. The EphB1 mRNA expression suppression was found to associate with a reduced overall survival rate and longer time to reach a complete remission. In this study, we defined the contribution of EphB1 to the DNA damage response system, which implies a tumor suppressor function for EphB1 in pediatric AML.
Rational design of combination therapies in AML

In the field of therapeutic target discovery, many researchers attempt to find new drug or drug combinations for the innovation of cancer treatments. From this thesis, it can be appreciated that dynamic signaling adaptation is an important factor that should be considered when applying drugs for cancer therapy. These can be discovered using high throughput methods e.g. protein arrays, kinase arrays and gene expression arrays, which allow the identification of possible bypass routes without a priori knowledge. Target specificity can be an important factor for the rational design of therapeutics, however, sometimes it’s also desirable to use “dirty” inhibitors that target for instance multiple RTKs. The broad spectrum availability of RTKs and ligands at the cancer cell surface may implicate that upstream targeting could require broad spectrum (“dirty”) inhibitors, while downstream targeting inhibitors might be designed with preferable high specificity. Two of these examples are discussed below.

Target specificity

In chapter 3 of this thesis, we aimed to combine VEGFC and PFKFB3 targeting approaches for more efficient eradication of AML blasts. By using this combination therapy approach in AML xenografts, we observed strong responses of endothelial cells to PFKFB3 inhibition because these cells are highly glycolytic. This partly disturbed the targeting effects of glycolysis inhibition on the AML blasts, which was partly restored by subsequent anti-VEGFC treatment. Therefore, the development of more specific glycolysis inhibitors that target leukemia blast should be developed to improve therapeutic responses. This can be achieved for instance by hematopoietic specific antibody based guidance of PFKFB3 inhibition. NBM stem and progenitor cells showed very low expression of the PFKFB3/PFKL axis and are therefore expected to be largely unaffected by this therapeutic strategy. The lack of target specific delivery of therapeutic compounds still causes therapy related toxicity that can be overcome by increasing their specificity. Antibody based delivery of kinase inhibitors is currently under development. Unfortunately these have not yet been evaluated extensively in clinical trials for cancer treatment, reflecting the complexity of target specificity.
Excluding FLT3 and KIT mutations, RTKs are not often affected by genetic mutations in AML (<1%). These RTKS have been identified as common resistance facilitating proteins for activating downstream survival pathways. Alternative bypass routes can be easily facilitated by RTKs, due to high receptor variability and subsequently the large availability of environmental growth factors. Therefore, the addition of broad spectrum inhibitors can be desirable for blocking possible bypass mechanisms. An important question in the field of dynamic kinome reprogramming is how do we define the cellular adaptations and how do we interfere at the level of kinase inhibition to enable a sufficient inhibition that will lead to the eradication of AML blasts without activating bypass RTK routes? Examples of these broad spectrum inhibitors are Sorafenib and Sunitinib that target cKit, FLT3, VEGFRs, PDGFRs all at once, or PTK787/ZK222584 (Vatalanib) targeting all except FLT3. These broad spectrum inhibitors are currently in phase III clinical trials for efficacy in many types of cancers (clinicaltrial.gov). In chapter 5 of this thesis, we investigated PTK787/ZK222584 (PTK) cellular adaptation opportunities in AML blasts. To uncover the adaptation route of PTK787 in AML blasts, we consulted a kinomics approach, which enabled the identification of sustained TRKA phosphorylation. Unexpectedly, we found that NGF and subsequent TRKA activation provides a survival benefit upon PTK787 inhibition in AML cells. Due to the fact that for example TRKA would easily end up in the background detection because of its low basal expression levels and targeting TRKA showed only minor anti-leukemic responses, it remains very challenging to identify these low expressed resistance pathways. Moreover, in the case of pharmacological inhibition of the PI3K/Akt/mTOR pathway it has been shown that while there was some degree of apoptosis, it was inadequate to eradicate AML blasts due to high IR, IGF-1R or PDGFR activity by facilitating therapeutic resistance. This is an important lesson from this study approach, which should be taken into account for future research. Screening cellular dynamics of activated signaling pathways upon kinase inhibition can identify initial background (RTK) signaling pathways that are able to facilitate therapeutic resistance during severe cellular stress.
Besides the fact that bypass mechanisms can facilitate opportunities for AML blasts for therapy resistance, also alterations in basic control systems can interfere with AML therapy responses. One of these basic systems is the DNA damage response system. This system is designed to detect and repair or discard genetic flaws. Although genetic alterations in this system are uncommon, the systems seems not fully operational in AML, since DNA damaged AML cells are not being repaired nor guided through programmed cell death. The DNA response system can be affected at many different levels of the pathway e.g. genetic, epigenetic, post-translational modifications, environmental interference and so on. Genetic alterations in TP53 are found in 8% of adult AML and 1% of pediatric AML.\textsuperscript{20,21} Translational suppression of the DNA damage response system can be observed for instance in AML patients with EphB1 repression through promoter hypermethylation, as we presented in this thesis, which prevents p53 from binding to the DNA for the activation of downstream programmed cell death responses. At the post-transcriptional level many altering interactions can occur. For instance, proteins that capture p53 and thereby prevent p53 to function normally to bind to the DNA for the transcription of cell cycle inhibiting proteins, which has been established for the MLL-ELL fusion proteins.\textsuperscript{22} Or overexpression of MDM2 that suppresses p53 activation, which allows to block its subsequent binding to the DNA.\textsuperscript{23} Also further downstream the alterations can occur through for instance by Bcl-2 overexpression, which prevents AML apoptosis.\textsuperscript{24} Different factors can contribute to suppression of the DNA damage response system in AML. The DNA damage response system is still poorly understood in AML. Although the DNA damage response is a very important control system that allows interfering with therapy sensitivity, the complexity is underscored by its multi-factorial interference.

We and others have found apparent DNA damage in AML patient samples. Interestingly, p53 phosphorylation was found to be significantly higher expressed in AML as compared to NBM. This finding suggests that the DNA damage response system is partially intact at least in a subgroups of AML patient samples and raises the question why these DNA damaged cells are then still able to survive and proliferate. In 1993, it was already established that Bcl2 was found to be regulated by growth factors IL2 and IL3.\textsuperscript{25} Through the years, more growth factors have been shown to increase Bcl2 expression e.g. FGF2, NGF and VEGF.\textsuperscript{26-28} Moreover, we observed that MLL-rearranged AML samples showed enhanced expression of Bcl2 peptide activity in comparison to NBM controls.
Subsequently, we showed that VEGFC was able to mediate apoptosis suppressing signaling via increased Bcl-XI expression. Anti-apoptotic Bcl-XL was previously shown to be associated with chemotherapy resistance in AML.\textsuperscript{16,28,29} AML blasts with high p53 expression are often defective in their programmed cell death pathways, which implicates that the addition of Bcl2/Bcl-XL inhibitors to conventional therapies would be beneficial to allow AML blasts to precede the induction of programmed cell death into apoptosis. Recent data shows high potential of Bcl-2 inhibitor ABT-199 in leukemia models and in clinical trials especially in IDH mutant leukemia patients (ASH abstract 2014, Konopleva et al., Blood 2014 Suppl;124:21 #118).\textsuperscript{30} These findings appoint the importance of investigating the DNA damage response system in more depth and especially in combination therapies, for the rational design of successful combination therapies.
Future Perspectives

Therapeutic intervention of AML heterogeneity

In this thesis, we attempted a broad-spectrum targeting approach to identify cellular resistance within the bulk of clones in individual AML patient samples. Few important remarks from this analysis can be appointed: 1) we observed patient-to-patient variability 2) we found unexpected background resistance pathways that solely interfered with AML survival when the AML cells encounter cellular stress. The question remains; how many inhibitors and subsequent resistance analysis need to be performed for optimal clinical responses? Importantly, one pitfall of the performed analysis is that they cannot distinguish therapeutic responses between different AML clones. There might be distinct genetic AML clones that rely on the same recurrent pathways for cellular resistance. In this case, we do not require a multi-clonal targeting approach. Nevertheless, with the focus on future perspectives, it would be ideally preferred to monitor all clones independently to build a library for clonal sensitivity to kinase inhibitors and their cellular adaptive bypass mechanisms for individual customized therapy. The complexity of AML involves the multi-factorial interaction between different leukemic clones and their specialized microenvironment, for which we would like to address potential opportunities in the ideal scientific world.

AML heterogeneity and therapeutic resistance

Currently, there is one study available that defined the best inhibitor combinations pre-clinically for therapeutic intervention and subsequently provided these inhibitors in an attempt to treat the AML patients. These so called pre-patient treatment screenings and subsequent kinase inhibition with FDA approved kinase inhibitors unfortunately only showed short-lasting cellular responses in AML. Moreover, an interesting observation with regards to clonal evolution is that the observed resistance was originated from the selection of pre-existing minor clones rather than the appearance of new clones, showing the utility of additional profiling after kinase inhibitor screens to identify targets of alternative routes. These results emphasize that eradication of major clones can be established and targeting those results in complete remissions, however, this will
allow minor previously subordinated clones to overgrow the bone marrow and facilitate relapses. Therefore, cellular responses to kinase inhibitors and their resistance facilitating clones is relevant during treatment to customize kinase inhibitor mixtures effective to target both major and minor clones. This might require extending the screening method of kinase inhibitors with proteomic and kinomic dynamic adaptation profiles. These findings can imply that the clonal evolution, which was found in the minority of the relapsed AML patients might be mainly due to therapy induced evolution, while the majority of the AML patients relapses is due to untargeted therapy resistant minor clones.32

AML clonal eradication strategy

As with recurrent mutations in AML that facilitate a block in AML differentiation and a proliferative advantage, there may also be recurrent signaling pathways for AML clonal survival and proliferation. First, these recurrent signaling pathway of different AML clones (when existing) need to be defined. Since we are currently unable to distinguish and isolate the different AML clones, we need to develop an identification strategy that enables to isolate the various AML clones from bone marrow aspirates. One implication could include the use of probe based flow cytometry for the isolation of specific groups of AML clones. Secondly, we need to perform an in dept analysis of the different AML clones to map their signaling alterations that facilitate their accelerating proliferation, their differentiation block, their metabolic self-sufficiency and their survival functions by using high throughput proteomic screens. In this way, we can define common recurrent signaling pathways of AML clones, for which we can predict and test their sensitivity towards kinase inhibitor combinations. Following upon this strategy, we need to perform an individualized high throughput checkerboard kinase inhibitor screen for the evaluation of kinase inhibitor combinations to optimize cellular responses and dosages. In this way, we can build a library that enables to define AML clonal sensitivity to kinase inhibitors. AML patients could then be diagnosed upon their clonal composition of the bone marrow for which a suitable kinase inhibitor cocktail can be customized. Thirdly, to overcome therapeutic resistance after the kinase inhibitor combination treatment, we need to identify and target alternative bypass mechanisms to optimize the combination therapies that intercept cellular resistance mechanisms. In the ideal situation this would be a high potential approach to eradicate AML clones and prevent relapses. By focusing
more on studying altered pathway activation and drug sensitivity of the different AML clones, we aim to achieve better therapeutic responses in the clinics. Moreover, from an environmental perspective, the described approach of clonal specific kinase inhibition ignores the interference of the bone marrow microenvironment that contributes to AML clonal protection and maintenance, which is an important mechanism for AML therapy resistance that should be taken into account. To tackle this problem it can be considered to perform the checkerboard kinase inhibitor screen in the presence of a defined growth factor/cytokine cocktail or in the presence of a stromal bone marrow feeder layer.
Conclusion

In conclusion, we are still far from the ideal situation to eradicate AML. However, we attempted to contribute to the understanding of cellular resistance in AML and adaptation opportunities for therapeutic intervention. Part of the studies that are described in this thesis show why currently used clinical FDA approved compounds were not successful so far in AML (MEK inhibition and PTK787 treatment). This thesis also provided combinations for therapeutic intervention that have not yet been investigated for their anti-leukemic activity in clinical trials (VEGFC/PFKFB3 inhibition). Five important major drivers of AML maintenance were appointed in this thesis as important characteristics that need to be tackled for therapeutic intervention: 1) AML proliferation advantage 2) AML differentiation block 3) AML metabolism for self-sufficiency, 4) altered AML DNA damage response system (EphB1 methylation), and 5) RTK availability for therapeutic resistance facilitating pathways.
Reference List
