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## Mechanisms of therapy resistance in acute lymphoblastic leukemia

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# Mechanisms of therapy resistance in acute lymphoblastic leukemia

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| Abstract<br><br>Acute lymphoblastic leukemia (ALL) is a highly aggressive pediatric cancer that can affect both B cells and T cells. The advent of new therapies has increased the cure rates for both B-ALL and T-ALL patients. However, some patients still experience relapse with a variable response to the treatment and display poor survival. Thus, identification of novel predictive biomarkers that can predict therapy resistance may help to stratify this group of patients. This could also aid in developing an effective treatment strategy.<br><br>Glucocorticoids are widely used along with the chemotherapeutic regimens for treating ALL patients. The response to glucocorticoids can predict long-term remission outcome. To understand the mechanisms of resistance to glucocorticoids, such as dexamethasone, we generated dexamethasone-resistant B-ALL cell lines in paper I. One such resistant cell line was found to possess increased FLT3 expression levels with *FLT3-ITD* and *FLT3-R845G* mutations that led to the activation of oncogenic RTK signaling. Further, second-generation FLT3 inhibitors, such as AC220 and crenolanib, suppressed this signaling both *in vitro* and *in vivo*.<br><br>We continued exploring the dexamethasone resistance mechanisms in paper II using a different approach. We observed that dexamethasone exposure caused upregulation of Aurora kinase and its various downstream effector kinases such as JAK, p38, mTOR, and S6K. These kinases lead to β-catenin stabilization through phosphorylation-dependent inactivation of GSK-3β either directly or indirectly. Indeed, we observed partial restoration of dexamethasone sensitivity with a combination of dexamethasone and inhibitors targeting either these kinases or β-catenin.<br><br>The expression of BCL2 varies in T-ALL depending on its stage of maturation, thereby T-ALL displays a heterogenous response to the BCL2-specific inhibitor venetoclax. We thus studied the mechanisms of venetoclax resistance using a panel of T-ALL cell lines in paper III. We observed that all the venetoclax-resistant T-ALL cell lines displayed non-universal changes in the expression of BCL2 family members and cancer stem cell markers, along with specific enrichment of cytokine signaling pathways. However, further investigations are warranted to identify additional mechanisms of venetoclax resistance in T-ALL.<br><br>Combination therapy is usually the choice of treatment to overcome monotherapy resistance. With this in mind, in paper IV we identified that inhibiting BCL2 by venetoclax synergizes with PLK1 inhibition by volasertib in T-ALL cell lines and PDX models. We observed that *BCL2L13* and *PMAIP1* genes get upregulated upon PLK1 inhibition, probably through transcriptional regulation by FOXOs in interaction with β-catenin. Thus, the pro-apoptotic functions exhibited by BCL2L13 and PMAIP1 probably synergize with BCL2 inhibition in T-ALL, with the help of sustained β-catenin levels. Moreover, we also identified upregulation of oxidative phosphorylation (OXPHOS) in T-ALL PDXs that didn't display synergy, which could be treated with a combination of venetoclax and oligomycin. However, additional experiments will be required to verify the above results. |
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# Mechanisms of therapy resistance in acute lymphoblastic leukemia

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Front cover by: Kinjal Shah
*Just as the lotus beautifully stems from muddy water, new treatments can emerge from the mucky root of cancer when it is understood better.*

*To all my loved ones*

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# List of papers

This thesis is based on the following papers:

I.  *Glucocorticoid-resistant B cell acute lymphoblastic leukemia displays receptor tyrosine kinase activation*.

Rohit A. Chougule, **Kinjal Shah**, Sausan A. Moharram, Johan Vallon-Christersson, and Julhash U. Kazi.

npj Genomic Medicine, 2019. 4(1):7 (p. 1-7).

II.  *The Aurora kinase/β-catenin axis contributes to dexamethasone resistance in leukemi*a.

**Kinjal Shah**, Mehreen Ahmed, and Julhash U. Kazi.

npj Precision Oncology, 2021. 5(1):13 (p. 1-12).

III.  *The mechanism of venetoclax resistance in T-cell acute lymphoblastic leukemia.*

**Kinjal Shah**, and Julhash U. Kazi.

*Manuscript*

IV.  *BCL2 inhibition synergizes with PLK1 inhibition in T-cell acute lymphoblastic leukemia.*

**Kinjal Shah**, Lina Al Ashiri, Mehreen Ahmed, Wondossen Sime, Katerina Bendak, Ramin Massoumi, Richard B. Lock, and Julhash U. Kazi.

*Manuscript*

# Abbreviations

| | |
|---|---|
| A1(BCL2A1/ | BCL2 related gene A1/ |
| BFL1) | BCL2–related gene expression in fetal liver |
| AKT | Serine/Threonine kinase, Protein kinase B |
| ALL | Acute lymphocytic leukemia |
| AML | Acute myeloid leukemia |
| AP-1 | Activator protein-1 |
| APC | Adenomatous polyposis coli |
| ATP | Adenosine triphosphate |
| B-ALL | B-cell acute lymphoblastic leukemia |
| BAD | BCL2/BCL-X-associated death promoter |
| BAK | BCL2 antagonist/killer |
| BAX | BCL2–associated X protein |
| BCL-W | B-cell lymphoma-w |
| BCL-XL | B-cell lymphoma-extra-long |
| BCL2 | B-cell leukemia/lymphoma 2 |
| BCL2L | BCL2 like protein |
| BCL6 | B-cell lymphoma 6 protein |
| BCR-ABL1 | Breakpoint cluster region protein-Abelson tyrosine-protein kinase 1 |
| BH | BCL2 homology |
| BID | BH-3 interacting-domain death agonist |
| BIK | BCL2-interacting killer |
| BIM | BCL2-interacting mediator of cell death |
| BIRC5 | Baculoviral IAP repeat containing 5 |
| BMF | BCL2-modifying factor |
| BOK | BCL2 homologous antagonist killer |
| CCLE | Cancer Cell Line Encyclopedia |

| | |
|---|---|
| CCND | Cyclin D |
| CD | Cluster of differentiation |
| CDK | Cyclin dependent kinase |
| CDKN | Cyclin-dependent kinase Inhibitor |
| CK1$\alpha$ | Casein kinase 1$\alpha$ |
| CLL | Chronic lymphocytic leukemia |
| CLP | Common lymphoid progenitor |
| CML | Chronic myeloid leukemia |
| CMP | Common myeloid progenitor |
| C-MYC | Cellular myelocytomatosis oncogene |
| CR | Complete remission |
| CREB | Cyclic AMP-responsive element binding protein |
| CREBBP | CREB binding protein |
| CRLF2 | Cytokine receptor-like factor 2 |
| DTP | Drug-tolerant persister |
| EBF1 | Early B cell factor 1 |
| EFS | Event-free survival |
| EGIL | European Group for the Immunological Characterization of Leukemias |
| EMT | Epithelial mesenchymal transition |
| ERK | Extracellular signal-regulated kinase |
| ETP-ALL | Early T-cell precursor acute lymphoblastic leukemia |
| ETV6- | ETS variant transcription factor 6 – |
| RUNX1 | Runt related transcription factor 1 |
| FBXW7 | F-box/WD repeat-containing protein 7 |
| FDA | Food and Drug Administration |
| FLT3 | FMS-like tyrosine kinase 3 |
| FOXO | Forkhead box-O |
| GDSC | Genomics of Drug Sensitivity in Cancer |
| GLOBOCAN | Global Cancer Incidence, Mortality and Prevalence |
| GR | Glucocorticoid receptor |
| GSEA | Gene Set Enrichment Analysis |
| GSK-3β | Glycogen synthase kinase-3β |

| HDAC | Histone deacetylase |
|------|---------------------|
| HOXA | Homeobox protein Hox-A |
| HRK | Harakiri |
| HSCs | Hematopoietic stem cells |
| HSCT | Hematopoietic stem cell transplantation |
| IGH | Immunoglobulin heavy locus |
| IKZF1 | IKAROS family zinc finger 1 |
| IL3 | Interleukin 3 |
| ITD | Internal tandem duplication |
| JAK | Janus kinase |
| KMT2A | Histone-lysine N-methyltransferase 2A |
| LDAC | Low-dose cytarabine |
| LMO2 | LIM domain only 2 |
| LSCs | Leukemic stem cells |
| LYL1 | Lymphoblastic leukemia derived sequence 1 |
| MAP-1 | Modulator of apoptosis-1 |
| MCL1 | Myeloid cell leukemia 1 |
| ML | Machine learning |
| MLL | Mixed lineage leukemia |
| MOMP | Mitochondrial outer membrane permeabilization |
| MPP | Multipotent progenitor |
| MPTP | Mitochondrial permeability transition pore |
| MRD | Minimal residual disease |
| mTOR | Mammalian target of rapamycin |
| NF-κβ | Nuclear factor-κβ |
| NK | Natural killer |
| NOTCH1 | Neurogenic locus notch homolog protein 1 |
| NOXA | NADPH oxidase activator |
| (PMAIP1) | (Phorbol-12-myristate-13-acetate-induced protein 1) |
| NR3C1 | Nuclear receptor subfamily 3 group C member 1 |
| NT5C2 | 5'-Nucleotidase, Cytosolic II |
| OXPHOS | Oxidative phosphorylation |
| PAX5 | Paired box 5 |

| PBD | Polo-box domain |
| PDX | Patient-derived xenograft |
| PI3K | Phosphoinositide 3-kinase |
| PIM1 | Proviral integration site for Moloney murine leukemia virus 1 |
| PLK | Polo-like kinase |
| PRoXE | Public Repository of Xenografts |
| PRPS1 | Phosphoribosyl pyrophosphate synthetase 1 |
| PUMA | p53 upregulated modulator of apoptosis |
| (BBC3) | (BCL2 binding component 3) |
| RB1 | Retinoblastoma-associated protein 1 |
| RBCs | Red blood cells |
| RTK | Receptor tyrosine kinase |
| S6K | Ribosomal protein S6 kinase |
| SAM | Significance Analysis of Microarrays |
| SOCS2 | Suppressor of cytokine signaling 2 |
| STAT5 | Signal transducer and activator of transcription 5 |
| T-ALL | T-cell acute lymphoblastic leukemia |
| TAL1 | T-cell acute lymphocytic leukemia protein 1 |
| TARGET | Therapeutically Applicable Research To Generate Effective Treatments |
| TCF3-PBX1 | Transcription factor 3 - Pre-B-Cell leukemia homeobox 1 |
| TLX | T cell leukemia homeobox |
| US | United States |
| WBCs | White blood cells |
| WHO | World Health Organization |
| WNT | Wingless-type MMTV integration site family |
| β-TrCP | Beta-transducin repeat-containing protein |

# Abstract

Acute lymphoblastic leukemia (ALL) is a highly aggressive pediatric cancer that can affect both B cells and T cells. The advent of new therapies has increased the cure rates for both B-ALL and T-ALL patients. However, some patients still experience relapse with a variable response to the treatment and display poor survival. Thus, identification of novel predictive biomarkers that can predict therapy resistance may help to stratify this group of patients. This could also aid in developing an effective treatment strategy.

Glucocorticoids are widely used along with the chemotherapeutic regimens for treating ALL patients. The response to glucocorticoids can predict long-term remission outcome. To understand the mechanisms of resistance to glucocorticoids, such as dexamethasone, we generated dexamethasone-resistant B-ALL cell lines in paper I. One such resistant cell line was found to possess increased FLT3 expression levels with *FLT3-ITD* and *FLT3-R845G* mutations that led to the activation of oncogenic RTK signaling. Further, second-generation FLT3 inhibitors, such as AC220 and crenolanib, suppressed this signaling both *in vitro* and *in vivo*.

We continued exploring the dexamethasone resistance mechanisms in paper II using a different approach. We observed that dexamethasone exposure caused upregulation of Aurora kinase and its various downstream effector kinases such as JAK, p38, mTOR, and S6K. These kinases lead to β-catenin stabilization through phosphorylation-dependent inactivation of GSK-3β either directly or indirectly. Indeed, we observed partial restoration of dexamethasone sensitivity with a combination of dexamethasone and inhibitors targeting either these kinases or β-catenin.

The expression of BCL2 varies in T-ALL depending on its stage of maturation, thereby T-ALL displays a heterogenous response to the BCL2-specific inhibitor venetoclax. We thus studied the mechanisms of venetoclax resistance using a panel of T-ALL cell lines in paper III. We observed that all the venetoclax-resistant T-ALL cell lines displayed non-universal changes in the expression of BCL2 family members and cancer stem cell markers, along with specific enrichment of cytokine signaling pathways. However, further investigations are warranted to identify additional mechanisms of venetoclax resistance in T-ALL.

Combination therapy is usually the choice of treatment to overcome monotherapy resistance. With this in mind, in paper IV we identified that inhibiting BCL2 by

venetoclax synergizes with PLK1 inhibition by volasertib in T-ALL cell lines and PDX models. We observed that *BCL2L13* and *PMAIP1* genes get upregulated upon PLK1 inhibition, probably through transcriptional regulation by FOXOs in interaction with β-catenin. Thus, the pro-apoptotic functions exhibited by BCL2L13 and PMAIP1 probably synergize with BCL2 inhibition in T-ALL, with the help of sustained β-catenin levels. Moreover, we also identified upregulation of oxidative phosphorylation (OXPHOS) in T-ALL PDXs that didn't display synergy, which could be treated with a combination of venetoclax and oligomycin. However, additional experiments will be required to verify the above results.

# Popular science summary

Blood comprises of different types of cells such as the red blood cells (RBCs), white blood cells (WBCs), platelets, natural killer cells and dendritic cells that arise from two distinct lineages; myeloid and lymphoid. WBCs are the soldiers of our body that fight various pathogens, thereby maintaining healthy immunity. Both the B and T lymphocytes arise from the lymphoid lineage and fight infections and various diseases, thereby keeping up with their WBC functions. When B and T cells proliferate continuously, they no longer can perform their normal functions. Instead, the healthy cells will be replaced with the abnormal cancerous cells. Thus, a person suffering from B-cell and T-cell acute lymphoblastic leukemia (ALL) might suffer from recurrent infections, bleeding, fever, etc.

Both B-ALL and T-ALL have been treated with various chemotherapeutic drugs along with glucocorticoids over the past years. Moreover, the advent of new targeted therapies has substantially improved the overall survival rate of ALL patients. However, some patients still relapse and show resistance to treatment. Therapy resistant clones containing certain genetic mutations might exist either from the start of the treatment, or they might be even developed during the course of treatment. Understanding the underlying mechanisms of therapy resistance might thus aid in identifying potential hits that can be specifically targeted, thereby reducing side effects. Thus, in this thesis, we have attempted to identify the mechanisms of dexamethasone resistance in B-ALL, along with identifying a potential mono or combination therapy to revert the resistance. Moreover, we have also tried to identify the mechanisms of venetoclax resistance in T-ALL, along with identifying a potential combination therapy.

In paper I, we generated dexamethasone-resistant B-ALL cell lines in the lab by prolonged culturing of these cells in increasing concentrations of dexamethasone. When these *in vitro* generated dexamethasone-resistant cells were analyzed, one out of three such cell lines displayed loss of the glucocorticoid receptor. Thus, dexamethasone would not be able to perform its function of inducing cell death, thereby contributing to resistance. This cell line also showed a difference in gene expression pattern as compared to its sensitive counterpart, and it even responded to inhibitors of receptor tyrosine kinases (RTKs). Upon further analysis, it was found to possess a strong tyrosine phosphorylation of the type III RTK FLT3, that contained 2 oncogenic mutations: internal tandem duplication (*ITD*) and a point mutation *R845G*. Both the mutations lead to constitutive activation of RTK

signaling with increased STAT5 levels, downstream of activated FLT3. This signaling was inhibited by the second generation FLT3 inhibitors; AC220 and crenolanib both *in vitro* and *in vivo*. Thus, if dexamethasone-resistant B-ALL patients are screened for the presence of FLT3 mutations, they could probably be treated using FLT3 inhibitors.

In paper II, we determined additional mechanisms of dexamethasone resistance using another B-ALL cell line model system. For this project, we exposed a B-ALL line to dexamethasone for a short term *in vitro* and even predicted dexamethasone resistance in ALL patients using deep learning *in silico*. After combining results from the two experiments, we observed that dexamethasone treatment induced the activity of various kinases, such as Aurora kinase, S6K, p38, JAK, mTOR, etc. Markers involved in the epithelial-mesenchymal transition (EMT) pathway were found to be enriched. EMT is usually involved in cancer metastasis, thereby linking to drug resistance. Since we even observed enrichment of the β-catenin pathway, we speculated the above kinases to stabilize its expression, thereby contributing to dexamethasone resistance. To confirm this, we first predicted synergy between dexamethasone and more than 1000 kinase inhibitors *in silico* using the same deep learning model in ALL patient samples. We then extrapolated the synergy observed between dexamethasone and kinase inhibitors *in vitro* in a dexamethasone-resistant cell line. In fact, we even detected synergy between dexamethasone and β-catenin inhibitors, thereby suggesting that the Aurora kinase and its various downstream effector kinases contribute to dexamethasone resistance, where β-catenin levels are maintained.

Thus, we observed diverse mechanisms of dexamethasone resistance in B-ALL, where some cells exhibited constitutive activation of FLT3 signaling, while others displayed activation of the Aurora kinase/β-catenin signaling axis.

T-ALL is the most aggressive pediatric malignancy, even though it occurs in only a quarter of ALL patients. The differentiation stage of T-ALL decides its dependency on either BCL2 or BCL-XL for survival. Thus, a heterogenous response of T-ALL cells to the BCL2-specific inhibitor venetoclax monotherapy might probably contribute to its resistance. We, therefore, aimed to determine the underlying mechanisms of venetoclax resistance in T-ALL in paper III. We couldn't identify any universal changes in all the venetoclax-resistant T-ALL cell lines. Instead, we observed the cell-line-specific expression of BCL2 family members and cancer stem cell markers. We also detected specific enrichment of cytokine signaling pathways. However, we might need to dig deeper to identify a common thread in all the venetoclax-resistant cell lines. For this, we would need to identify the mutational and the phosphorylation status of BCL2 that might hamper venetoclax binding, thereby contributing to resistance.

The most common solution to the problem of monotherapy resistance is the use of combination therapy. In paper IV, we identified that T-ALL patients that were

predicted to be venetoclax-resistant exhibited enrichment in Aurora kinase and PLK1 pathways. Moreover, PLK1 inhibitors enhanced the efficacy of a BCL2 family inhibitor, navitoclax. Thus, PLK1 was chosen as the target to be inhibited in combination with BCL2. Indeed, PLK1 inhibition by volasertib synergized with BCL2 inhibition by venetoclax in a panel of T-ALL cell lines *in vitro*, PDXs *ex vivo,* and a mouse model *in vivo*. We observed induction of *BCL2L13* and *PMAIP1* upon PLK1 inhibition by volasertib. The transcription of these genes is probably mediated by FOXOs in combination with β-catenin, as we observed sustained β-catenin levels. Moreover, we also observed enrichment of the arachidonic acid metabolism pathway, which is involved in stabilizing β-catenin. Thus, regulation of BCL2 family proteins along with contributions from fatty acid metabolism pathway were identified as the possible mechanisms behind synergy. Apart from this, oxidative phosphorylation (OXPHOS) was upregulated in T-ALL PDXs not displaying synergy. But inhibiting it along with venetoclax displayed synergism. However, additional experiments might be beneficial to prove FOXOs-β-catenin interaction, along with the role of proteins induced by *BCL2L13* and *PMAIP1* genes in T-ALL.

Thus, we observed diverse cell-line specific mechanisms of venetoclax resistance in T-ALL. To overcome venetoclax resistance, we even identified a possible therapy in combination with volasertib that inhibits PLK1. We even studied the underlying mechanisms of synergy in T-ALL using cell lines and PDX samples. However, additional experiments shedding more light on identifying the mechanisms of venetoclax resistance, along with studying the underlying mechanisms of synergy might be extremely valuable.

# Introduction

## Cancer

Estimates of the global cancer statistics in 2020 are provided by the GLOBOCAN database that tracks the incidence and mortality for 36 cancers in 185 countries. According to this study, an estimated 19.3 million new cases of cancer and almost 10 million deaths from cancer occurred in 2020. Moreover, based on the global demographic projections, a 47% rise in the global cancer burden is predicted in 2040 with approximately 28.4 million cases as compared to 2020. Cancer is thus an important contributor to the ever-increasing morbidity and mortality rates worldwide, irrespective of the level of human development [1].

Most of the cancers during their development acquire a set of functional capabilities through various mechanistic strategies. The following are the eight hallmarks of cancer: replicative immortality, evasion of apoptosis, self-sufficiency in growth signals, insensitivity to growth suppressors, sustained angiogenesis, tissue invasion and metastasis, ability to evade the immune system, and deregulate the energy metabolism. Moreover, genomic instability and tumor-promoting inflammation are the characteristics of cancer that enable tumors to acquire the above hallmarks [2, 3]. Cancer is thus characterized by uncontrolled growth and proliferation. Therapeutic targeting of each of these acquired capabilities in cancer with various inhibitors might thus hamper the growth and proliferation of cancer cells.

### Tumor heterogeneity

Tumors exhibit marked histological and functional heterogeneity that leads to the generation and classification of discrete tumor subtypes. These subtypes exhibit heterogeneity in terms of their morphology, genetic lesions, expression of specific markers, proliferative index and therapeutic response [4]. Intertumoral heterogeneity refers to heterogeneity among tumors in between patients, while intratumoral heterogeneity refers to heterogeneity among tumors within the same patient [5]. Extrinsic mechanisms like interactions of tumor cells with the stromal microenvironment can also contribute to tumor heterogeneity [6]. Besides, cancer is a dynamic disease that generally becomes more heterogenous during its progression. Heterogeneity in tumors fuels therapeutic resistance; and thus, its accurate assessment is essential for the development of effective therapies [5].
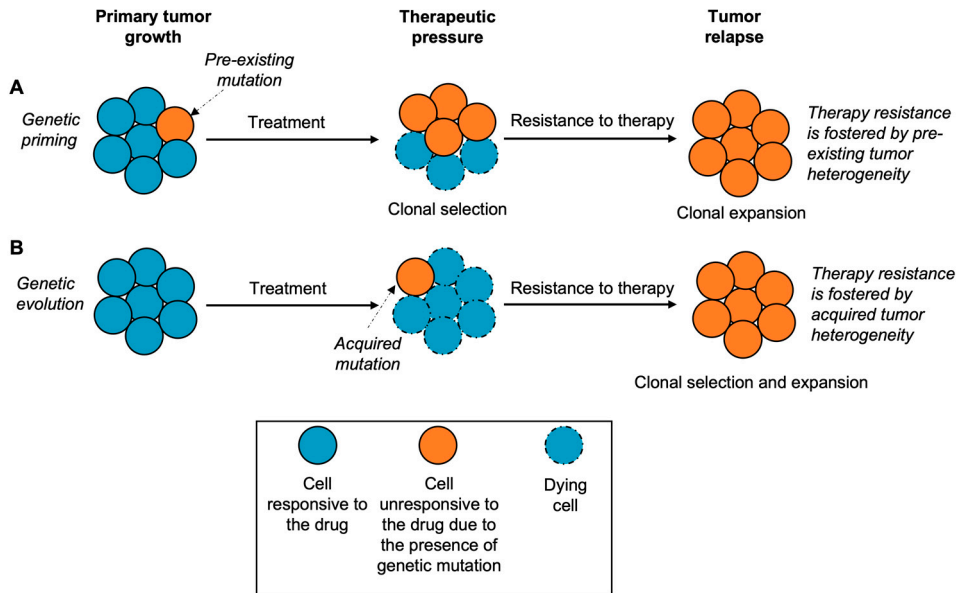
## Mechanisms of therapy resistance

A growing tumor is normally subjected to hypoxic, metabolic, and nutritional pressure. While some clones will be eradicated when challenged with this pressure, others containing certain mutations will be able to survive and grow in clonal size. Bulk tumor sequencing primarily detects mutations within the dominant clones of a tumor population. Thus, cancer is said to have undergone sequential genetic evolution under therapeutic pressure. In contrast, high-resolution single-cell sequencing of cancerous cells suggests that different clones that emerge throughout the treatment might already exist at the beginning, manifesting themselves in different clonal sizes. Thus, cancer is said to be evolved through non-genetic mechanisms of transcriptional and metabolic adaptation to the therapeutic pressure with no new genetic mutations. Therapeutic resistance can thus occur through both the genetic and non-genetic mechanisms as identified by different technologies [7].

Tumor evolution leading to therapy resistance has been mostly viewed through genetic lens [7]. Certain mutant cells are passively selected by therapy over time due to the 'Darwinian' selection of such cells. These cells carry a genetic mutation that is acquired by chance in low allele frequencies either before or during the treatment. This leads to a shift in clonal composition over the course of treatment [8]. Characterizing tumors at multiple timepoints during the treatment is, therefore, necessary to accurately capture the genomic composition during clonal evolution [5]. Thus, tumors can either be genetically primed (Figure 1A) or they genetically evolve (Figure 1B) to undergo therapy resistance.

Non-genetic resistance mechanisms such as transcriptional priming and transcriptional adaptation involve changes in the chromatin structure and function. Transcriptional priming leads to the expression of a particular transcriptional program that confers intrinsic resistance to a particular drug. On the other hand, transcriptional adaption allows some cancer cells to rapidly adapt and escape the therapeutic pressure by rewiring their gene expression leading to the acquisition of a particular transcriptional program that offers a selective advantage. These mechanisms substantially contribute to intratumor heterogeneity that can ultimately lead to the development of drug resistance [7].

When cancer cells are treated with a particular therapy, some cells will succumb to the treatment while others are spared by it. This population of residual malignant cells is clinically defined as the "minimal residual disease" (MRD), which forms a reservoir from which fully resistant tumor cells can emerge. A small population of slow-cycling drug-tolerant cells known as the "drug-tolerant persister" (DTP) cells constitutes the residual disease, where non-genetic mechanisms sustain their survival under therapy [9]. Upon withdrawal of the drug, these persister cells can lead to tumor relapse by reinitiating cell cycle progression. However, this progeny of cells is equally sensitive when rechallenged with initial therapy [10, 11]. This represents a transient or a reversible non-genetic mechanism of resistance (Figure 2A).

**Figure 1. Genetic mechanisms of therapy resistance.** (A). A pre-existing tumor heterogeneity due to the presence of a genetic mutation in a cancer cell primes it and confers intrinsic resistance to survive the therapeutic pressure. (B). Alternatively, a mutation in a cancer cell can also be acquired during the treatment that confers resistance to therapy. Both the genetic mechanisms lead to the selection and expansion of clones containing these mutations, thereby contributing to therapy resistance and tumor relapse (Adapted from [7]).

DTP cells are characterized by a slow cell cycle with distinct transcriptional program and epigenetic features, altered metabolism, immune evasion, and resistance to apoptosis. Increased cellular plasticity and heterogeneity underlie all these features and this provides multiple possibilities of rewiring the transcriptional program that favors a selective advantage [9]. The ability of a cell to switch its phenotype by altering its lineage commitment/differentiation status is known as cellular plasticity [12]. Thus, cancer cells can exhibit cellular plasticity in terms of "de-differentiation" or "trans-differentiation", where the former triggers the cell to acquire stem or progenitor cell properties, while the latter triggers the cell to acquire features belonging to a different lineage. Moreover, cellular plasticity is a reversible process and is not necessarily driven or associated with specific genetic modifications [13], albeit it can be induced by drug treatment or interactions with the tumor microenvironment. However, cell plasticity can also influence cancer evolution through transcriptional and/or epigenetic changes that are heritable [14]. Furthermore, diverse cancer types exhibit 'Lamarckian' induction, where a small proportion of cancer cells transiently acquire a drug-refractory phenotype through epigenetic modifications [15]. Thus, some of the DTP cells might possess stem-cell-like features due to de-differentiation, where increased stemness leads to increased tumor re-initiating capacity. DTP cells can also possibly harbor metabolic, epigenetic, and transcriptional reprogramming leading to tumor progression. The

tumor clones generated from such a plethora of non-genetic adaptive mechanisms might thus belong to either the same lineage or exhibit lineage infidelity/trans-differentiation that show resistance to therapy, thereby stably inheriting the non-genetic mechanisms of resistance [7] (Figure 2B).
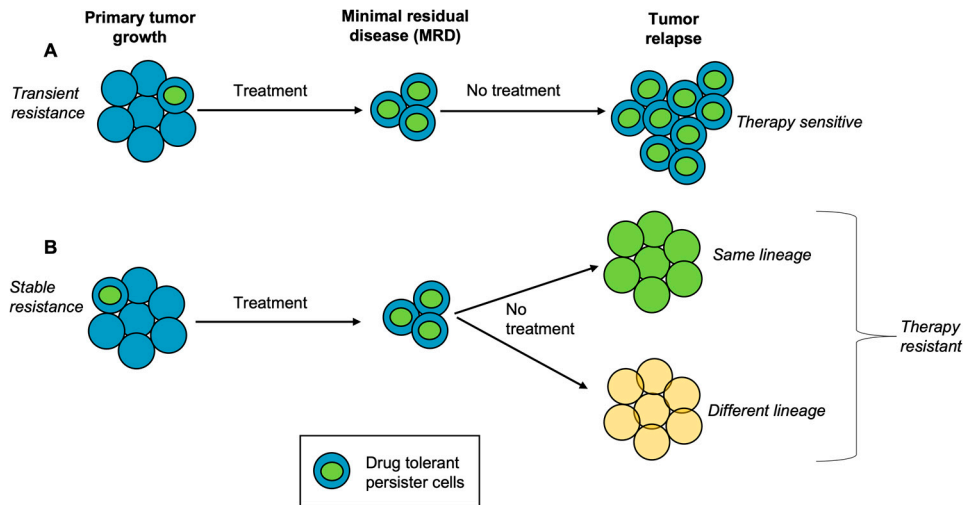


**Figure 2. Non-genetic mechanisms of therapy resistance.** Most of the cancer cells initially respond to treatment, except a few malignant ones that survive the drug exposure. These cells comprise the minimal residual disease (MRD). Drug-tolerant persister (DTP) cells constitute MRD after surviving the initial treatment. (A). Upon treatment withdrawal, DTP cells proliferate and lead to tumor relapse. However, the progeny remains sensitive to the initial therapy, thereby possessing a transient non-genetic mechanism of resistance. (B). Upon treatment withdrawal, DTP cells can instead undergo rapid transcriptional and metabolic reprogramming to generate a tumor population that either belongs to the same lineage or displays a lineage switch, thereby displaying cellular plasticity. Both the clones exhibit resistance to the initial therapy, thus stably inheriting the non-genetic mechanism of resistance (Adapted from [7]).

Diverse genetic and non-genetic mechanisms can collectively contribute to therapy resistance in most cancers. Thus, strategies that permit early detection of tumors along with better identification of their intrinsic properties would assist in the development of effective therapies for preventing and treating tumor relapse. This would ultimately aid in the improved overall survival of patients.

# Hematopoiesis and Leukemia

## Hematopoiesis

Hematopoiesis is a hierarchical process of generating the entire repertoire of blood cell lineages from hematopoietic stem cells (HSCs), that together constitute the hematopoietic tree [16, 17] (Figure 3). Due to the stem cell property, HSCs undergo self-renewal to maintain their stem cell pool and even differentiate into different types of mature blood cells. In mammals, HSCs reside in a specific microenvironment within the bone marrow known as the 'Hematopoietic Niche', which is composed of stromal cells and an extracellular matrix containing fibronectin, collagen, and proteoglycans. Specific signals emanating the niche regulate HSC maintenance [17]. Thus, hematopoiesis encompasses erythropoiesis, thrombopoiesis, and leukopoiesis, which involves the production of erythrocytes (red blood cells), thrombocytes (platelets), and leukocytes (white blood cells such as granulocytes and agranulocytes) respectively [16]. These cells can proliferate extensively if the mechanisms that control their production are disrupted. Hematopoietic malignancies are thereby classified according to the type of cells involved [18].

## Leukemia

### Introduction

The term 'Leukemia' is originally derived from the Greek words 'leukos' and 'haima', which means 'white' and 'blood' respectively [19]. Clonal expansion of leukocytes in the bone marrow is referred to as 'Leukemia', where cells of the affected lineage circulate in blood in elevated amounts [20]. The composition of the healthy bone marrow is thus ruined as the normal functional blood cells are replaced with the malignant ones, thereby leading to clinical symptoms such as anemia, frequent infection, easy bleeding, weakness, weight loss, fatigue, etc. [18]. The accurate cause of developing leukemia is unknown in most of the cases, albeit there are various factors that influence the risk of developing the disease. These include age, ethnicity/gender, inherited syndromes, cigarette smoking, obesity, infection by

certain viruses, exposure to benzene, household pesticides, chemotherapy, and ionizing radiation [18, 21]. Leukemia comes under the list of 15 most common cancers in the world, where its incidence and mortality rates are higher in countries with high to very high human development index (HDI) as compared to those with low to medium HDI [1].
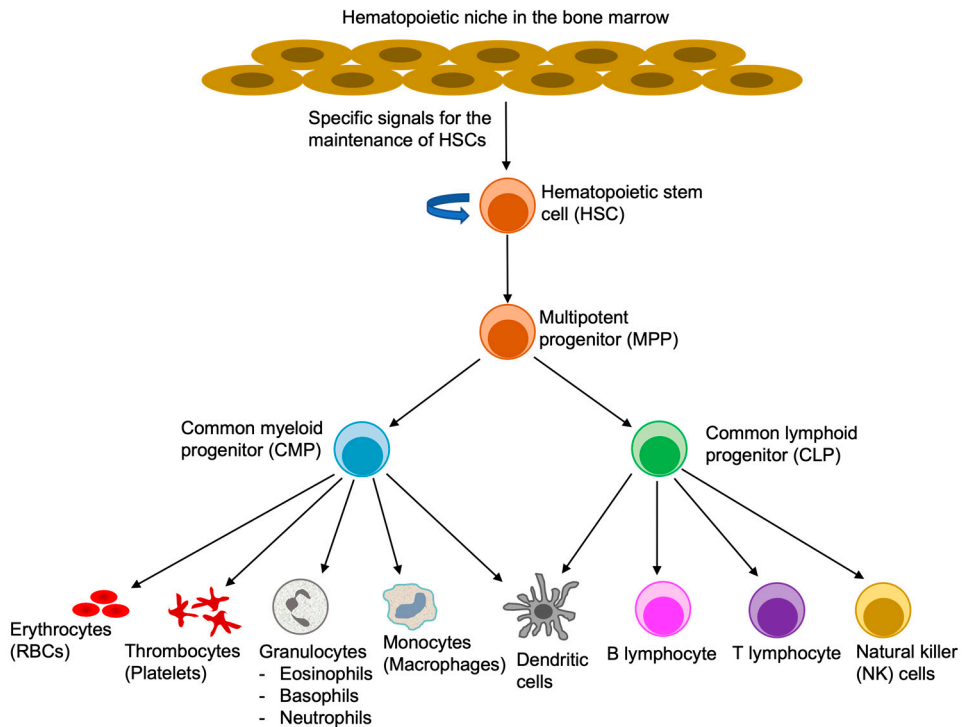


**Figure 3. Schematic overview of the hematopoietic tree.** Hematopoietic stem cells (HSCs) reside in the hematopoietic niche of adult bone marrow; where specific signals from the niche aids in HSC maintenance. HSCs undergo self-renewal and differentiate to a multipotent progenitor cell (MPP), that further differentiates into common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) cells. These progenitor cells produce all the mature blood cell types that together constitute the immune system (Adapted from [17]).

## Classification

Leukemia is classified into four subtypes depending on the progression of disease as well as the type of white blood cells affected. Progression of the disease can be acute or chronic. Acute leukemia is characterized by a sudden uncontrolled proliferation of the immature cells, known as "blasts", thereby resulting in rapid onset of symptoms that can be fatal if left untreated. In contrast, chronic leukemia is a slow-growing continuous disease of mature cells and may take years to develop symptoms. In leukemia, the type of white blood cells affected can either belong to the myeloid or lymphoid lineage, thus giving rise to myeloid and lymphocytic

leukemia in the bone marrow respectively. Thus, altogether, there exist four major subtypes of leukemia: Acute Myeloid Leukemia (AML), Acute Lymphocytic Leukemia (ALL), Chronic Myeloid Leukemia (CML) and Chronic Lymphocytic Leukemia (CLL) [18, 22].

## Diagnosis and Treatment

Accurate identification and classification of leukemia into distinct subtypes is extremely essential for proper clinical intervention. Leukemia can be diagnosed by a combination of several invasive and non-invasive techniques. These include a physical examination of the patient, performing blood tests along with peripheral blood smear, assessing bone marrow aspirate or biopsy, cytogenetic analysis, molecular testing to evaluate abnormalities in the DNA, and immunophenotyping using flow cytometry [18, 21]. Once leukemia is diagnosed, several approaches can be used either as a monotherapy or combination therapy to treat patients depending on the leukemia subtypes and patient-specific factors. These treatments include chemotherapy, radiotherapy, immunotherapy (monoclonal antibodies), tyrosine kinase inhibitors and hematopoietic stem cell transplantation (HSCT) [18, 22].

# Acute lymphoblastic leukemia (ALL)

## Introduction

Acute lymphoblastic leukemia (ALL) constitutes the most frequent pediatric malignancy of lymphoid progenitor cells, and about 80% of cases occur in children and 20% in adults [23]. The incidence curve for ALL is distinctly bimodal or U-shaped, with the highest occurrences in children between the ages of 2-5 and rising again in adults after the age of 40 [18, 20, 24]. The incidence rate of childhood ALL is higher in boys as compared to girls (male:female ratio is 55%:45%) [25].

## Origin of acute leukemia

Various endogenous and exogenous exposures, random chance event, and inherited genetic susceptibility cumulatively contribute to the vulnerability of the stem or progenitor blood cells to transforming events either *in utero* leading to the initiation of ALL or with its subsequent progression postnatally and clonal evolution, or both [24]. Chromosomal translocations disrupting genes involved in the regulation of normal hematopoiesis and lymphoid development can arise before birth. For example, the fusion of two hematopoietic transcription factors *ETV6-RUNX1* has been observed in a quarter of pediatric ALL patients. However, this rearrangement

occurs *in utero*, i.e. years before developing leukemia, and therefore it alone is inadequate to generate leukemia in experimental models [26]. *KMT2A* (*MLL*) rearrangement has also been observed *in utero* [27]. Thus, although such rearrangements might be important in initiating leukemia and also widely used in diagnosis and risk stratification, they alone are insufficient to explain the multi-step process of leukemogenesis [26].

## Survival statistics

Due to several factors, the prognosis of pediatric ALL patients has greatly improved over the last years with a 5-year overall survival rate approaching 90%, whereas it is only 30-40% in adult ALL patients. Moreover, treating adult ALL is challenging as these patients are faced with higher risk factors at diagnosis, along with increasing age and other comorbidities that often result in dose reductions [28].

## Treatment and Relapse

Induction of remission, intensification or consolidation and continuation or maintenance represents the three main phases of chemotherapy to treat ALL that lasts for approximately 2-2.5 years, where a combination of various chemotherapeutic drugs are used at different doses. Some of these include the glucocorticoid dexamethasone, vincristine, asparaginase, anthracycline, methotrexate and mercaptopurine [24]. ALL consists of clonal heterogeneity and genetic alterations in leukemic cells may occur either during therapy or exist as minor subclones before the start of treatment. Examples include mutations in *CREBBP* that confer resistance to glucocorticoids [29], and mutations in *NT5C2* and *PRPS1* that confer resistance to thiopurines [30-32]. Thus, relapsed patients usually exhibit resistance to standard chemotherapy. Patients who relapse or belong to the high-risk group due to certain genomic aberrations are mostly treated with allogeneic hematopoietic stem cell transplantation. Further, survivors of ALL would have to endure the long-term toxic effects of chemotherapy, and around 1-2% patients might even succumb to it [25]. Identifying mutations that contribute to therapy resistance and relapse is therefore essential in order to find an alternative targeted therapy that can achieve a better treatment outcome and even mitigate the toxic effects of chemotherapy.

# B-cell and T-cell acute lymphoblastic leukemia (B-ALL and T-ALL)

## Introduction

Depending on the type of cells affected, ALL can be categorized as either B-ALL or T-ALL that occurs with a frequency of about 75-85% and 15-25% respectively [23, 33, 34]. Stepwise accumulation of genetic and epigenetic aberrations in immature cells (progenitor or precursor) of both the B and T lineages leads to their differentiation arrest. This is followed by uncontrolled proliferation of these lymphoid blasts and their accumulation in the bone marrow and thymus respectively. Both the B and T leukemic blasts then migrate from their sites of origin to peripheral blood and ultimately metastasize to various organs such as lymph nodes, spleen, liver, and central nervous system, thereby hijacking the normal hematopoietic system [35, 36].

## Classification

World Health Organization (WHO) recommends that at least 20% of blasts should be detected in the blood or bone marrow for diagnosis of the majority of acute leukemias, and suitable testing must be performed in order to detect any genetic anomalies [37]. Thus, the WHO classification system is based on the morphologic, clinical, prognostic, immunophenotypic, cytogenetic and molecular genetic findings. Based on the recurrent cytogenetic abnormalities, B-ALL is subdivided into nine subtypes that possess unique prognostic, phenotypic, and clinical features. B-ALL cases without any specific genetic abnormalities are classified as not otherwise specified (Table 1). In contrast, assays to measure non-overlapping genetic subgroups of T-ALL that can be accurately matched to the differentiation stages are not yet standardized and the prognostic implications can be controversial. Thus, these subgroups are not formally classified by WHO. However, WHO has recognized the early T-cell precursor acute lymphoblastic leukemia (ETP-ALL) subtype that has a unique genetic makeup and immunophenotype [38].

Additionally, leukemias are also classified according to the European Group for the Immunological Characterization of Leukemias (EGIL) classification that is mostly based on the immunophenotype of leukemia [33, 39]. B-ALL and T-ALL are thus classified into four clinically relevant biological subgroups based on the expression of certain markers (Table 1).

**Table 1: Classification of ALL.** Classification of B-ALL and T-ALL based on the genetic abnormalities and immunophenotype. The EGIL subgroups express additional markers, but only the most relevant ones are displayed. (cCD22 = cytoplasmic CD22; cCD3 = cytoplasmic CD3; sCD3 = surface CD3).

---

**WHO classification** [37]

**B-lymphoblastic leukemia/lymphoma**

***B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities***
B-lymphoblastic leukemia/lymphoma with hyperdiploidy
B-lymphoblastic leukemia/lymphoma with hypodiploidy
B-lymphoblastic leukemia/lymphoma with t(12;21)(p13.2;q22.1); *ETV6-RUNX1 (TEL-AML1)*
B-lymphoblastic leukemia/lymphoma with t(v;11q23.3); *KMT2A (MLL)* rearranged
B-lymphoblastic leukemia/lymphoma with t(9;22)(q34.1;q11.2); *BCR-ABL1*
B-lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3); *TCF3-PBX1 (E2A-PBX1)*
B-lymphoblastic leukemia/lymphoma with t(5;14)(q31.1;q32.3); *IGH/IL3*
B-lymphoblastic leukemia/lymphoma with intrachromosomal amplification of chromosome 21 (iAMP21)
B-lymphoblastic leukemia/lymphoma with chromosomal translocations involving cytokine receptors or tyrosine kinases (BCR-ABL1–like)

***B-lymphoblastic leukemia/lymphoma, NOS (not otherwise specified)***

**T-lymphoblastic leukemia/lymphoma**
Early T-cell precursor acute lymphoblastic leukemia (ETP-ALL)

---

**EGIL classification** [33, 39]

**B-lymphoblastic leukemia**

| | |
|---|---|
| Early (pro/pre-pre) B-ALL (B-I) | CD19, cCD22, CD79a |
| Intermediate (common) B-ALL (B-II) | CD19, cCD22, CD79a, CD10 |
| Pre B-ALL (B-III) | CD19, cCD22, CD79a, CD10, cytoplasmic heavy µ chain, |
| Mature B-ALL (B-IV) | CD19, cCD22, CD79a, CD10, cytoplasmic heavy µ chain, surface immunoglobulin light chain |

**T-lymphoblastic leukemia**

| | |
|---|---|
| Pro-T-ALL (T-I) | cCD3, CD7 |
| Pre-T-ALL (T-II) | cCD3, CD7, CD5/CD2 |
| Cortical-T-ALL (T-III) | cCD3, sCD3+/-, CD1a |
| Mature-T-ALL (T-IV) | cCD3, sCD3, CD1a- |

## Survival statistics

<u>B-ALL</u>: Despite similar complete remission (CR) rates in both the groups, around 80-90% pediatric and only 40% adult B-ALL patients exhibit long-term survival [40].

<u>T-ALL</u>: More than 85% of T-ALL cases have a 5-year event-free survival, while around 20% and 40% of pediatric and adult T-ALL patients still relapse and develop therapy resistance [41, 42].

## Pathobiology

Distinct ALL subtypes such as B-ALL and T-ALL are characterized by a collection of chromosomal rearrangements and somatic genetic alterations. These include

insertions, deletions and translocations of genes which aim at de-regulating the lymphoid development, and even aneuploidy [24-26] (Figure 4).

B-ALL: B-ALL is often characterized by hyperdiploidy (non-random gain of at least 5 chromosomes), hypodiploidy, and recurring chromosomal translocations leading to the generation of a variety of fusion partners such as *ETV6-RUNX1*, *TCF3-PBX1*, and *BCR-ABL1* [24]. *MLL*-rearranged ALL is aggressive and harbors very few genetic alterations [43, 44], whereas ALL possessing *ETV6-RUNX1* and *BCR-ABL1* translocations typically harbor more alterations. *PAX5, IKZF1, and EBF1* are important transcriptional regulator genes involved in B-cell development, and these are most frequently altered in more than two-thirds of B-ALL cases [43, 45]. CRLF2 serves as the receptor for thymic stromal lymphopoietin, and its rearrangement results in novel fusions; *IGH@-CRLF2* and *P2RY8-CRLF2* in 8% childhood cases [46, 47]. These rearrangements increase the expression of CRLF2 on leukemic lymphoblasts. The gene expression profile of *BCR-ABL1*-like B-ALL is similar to that of the *BCR-ABL1* disease. However, this subtype lacks *BCR-ABL1* expression, displays frequent *IKZF1* alteration and poor outcomes [45, 48]. Moreover, around half of BCR-ABL1-like cases possess *CRLF2* rearrangements and *JAK* mutations [24]. Intrachromosomal amplification of chromosome 21 (iAMP21) constitutes 2% cases, where at least three copies of *RUNX1* are gained [49].

Pediatric B-ALL patients (25-30%) with *ETV6-RUNX1* and hyperdiploidy (>50 chromosomes) are associated with a favorable prognosis, while those with rearrangement of *MLL, BCR-ABL1,* and *CRLF2*, *BCR-ABL1*-like ALL, hypodiploidy (<44 chromosomes), and intrachromosomal amplification of chromosome 21 (iAMP21) are all associated with unfavorable prognosis [25].

T-ALL: Most of the pediatric T-ALL cases are characterized by deregulation of three core pathways: expression of T-cell transcription factors, NOTCH1/MYC signaling, and cell cycle control [50]. Deregulation of T-ALL transcription factors such as *TAL1, TLX1, TLX3, LMO2/LYL1, HOXA*, etc. occurs in >90% cases due to their rearrangement with the T-cell antigen receptor loci, thereby generating transcription factor-driven subtypes of T-ALL [24, 51, 52]. NOTCH1 is a critical transcription factor in T-ALL development, and aberrant activations in *NOTCH1* have been observed in >75% cases leading to constitutive NOTCH1 signaling [53]. Moreover, loss-of-function mutations in the negative regulator *FBXW7* are observed in 25% cases, that together with activating mutations in *NOTCH1* leads to uncontrolled growth, partly through increased *MYC* expression [54, 55]. Deletion of the tumor suppressor loci, *CDKN2A/CDKN2B* occurs in 80% cases, while that involving *CDKN1B, RB1, or CCND3* are less common [52, 56]. T-ALL also frequently involves deregulation of other transcription factors and epigenetic modifiers [52]. Moreover, PI3K-AKT and JAK-STAT signaling pathways are frequently activated in T-ALL [57, 58]. ETP-ALL belongs to a high-risk subgroup of immature T-ALL that arises due to a block in the earliest stages of T cell differentiation. Such patients can be possibly treated with myeloid-directed

therapies since this subgroup possesses a similar transcriptional profile as that observed in myeloid progenitors and HSCs [38, 59]. This subtype is associated with unfavorable prognosis [25].
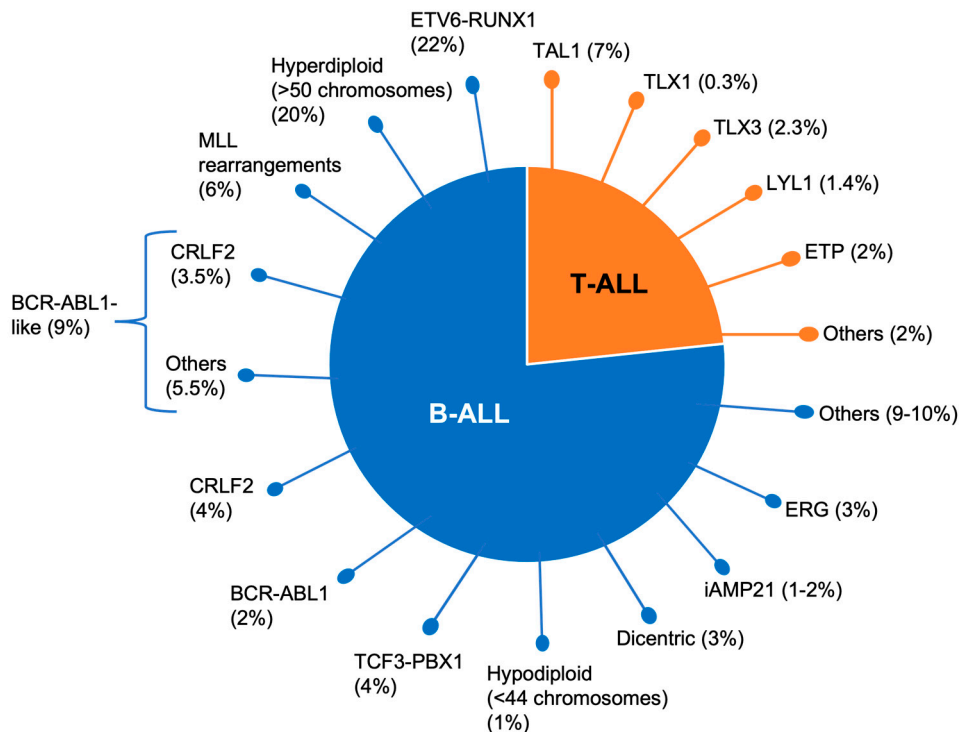


**Figure 4. Frequency of cytogenetic abnormalities in pediatric ALL.** The diagram represents the frequency of cytogenetic subtypes in B-ALL and T-ALL. ETP = Early T-cell precursor; iAMP21 = intrachromosomal amplification of chromosome 21 (Adapted from [24] and [26]).

# Treatment

Apart from chemotherapy, usage of various specific inhibitors has been implicated for treating the cytogenetic subtypes of B-ALL and T-ALL. Some of these include BCL2, ABL1, JAK, PI3K, and HDAC inhibitors [50]. Allogeneic HSCT is recommended for patients that belong to the following risk groups: BCR-ABL1+ ALL, BCR-ABL-like ALL, KMT2A-rearranged ALL, ETP-ALL, and ALL with low hypodiploidy and complex cytogenetics [60]. Further, the development of various monoclonal antibodies, tyrosine kinase inhibitors, cell signaling inhibitors, proteosome inhibitors, hypomethylating agents, and chimeric antigen receptor (CAR) T cells as therapies has immensely contributed to the overall progress in treating ALL patients [23, 50, 60].

# Prediction of cancer therapy

## Introduction

Over the years, cure rates for various cancer patients have significantly improved due to the advent of targeted therapies. However, different patients respond differently due to intertumor and intratumor heterogeneity, and thereby relapse due to treatment resistance. It would thus be highly beneficial to know right from the beginning whether a patient will respond to a particular treatment. This motivated the idea of predicting drug responses in cancer patients using artificial intelligence, such as machine learning, where patients can be better matched to drugs.

Machine learning (ML) is widely used in cancer research, where a large amount of available biological data (big data) is used to build a model that can predict patient's responses to either mono or combination therapies [61, 62]. The results so generated can then be verified *in vitro* and *in vivo* using cancer cell lines, PDXs or patient samples. Thus, precision medicine would not only aim to increase the overall survival of cancer patients, but also reduce unwanted side effects.

## Developing computational models for predicting drug responses

There are four basic steps in developing computational models for predicting drug responses: Selecting datasets and processing the input data, training the model, testing the model, and assessing the model for drug prediction on independent datasets with unknown drug sensitivity (Figure 5).

### Selecting datasets and processing the input data

Drug response prediction models are typically trained on publicly available datasets that are generated by various large research consortia [63]. High quality biological data are widely available for a plethora of cell lines in various pharmacogenomic data resources [62]. Some of them include the Cancer Cell Line Encyclopedia (CCLE) [64], the Genomics of Drug Sensitivity in Cancer (GDSC) [65], NCI-60

[66], and the PharmacoDB [67]. Baseline genomic and transcriptomic data (data from untreated samples) for various cancerous cell lines are covered by these databases. Moreover, DrugComb [68] and DrugCombDB [69] contain drug combination data that is manually curated from different studies [62]. Pharmacogenomic data for PDXs are also available in PDX finder [70], and PRoXE [71] to name a few. Pre-processing of data is an important step, that includes data selection, feature selection, noise filtering, imputation of missing values, and data normalization [62].
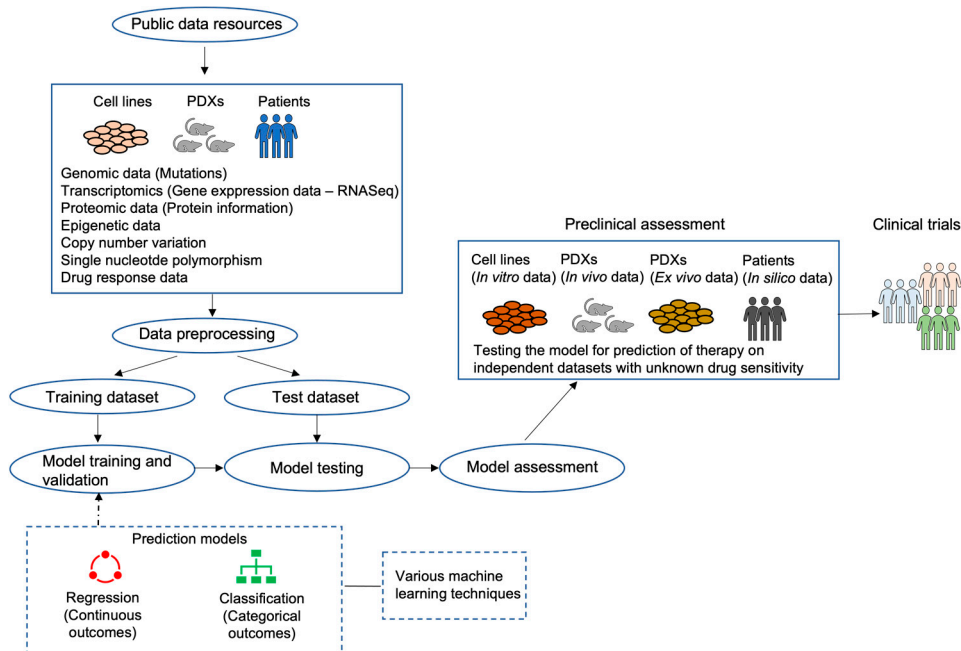


**Figure 5. Workflow in the development of machine learning prediction model.** Biological data from pharmacogenomic resources is pre-processed before being used for training the model using various machine learning algorithms. Thereafter, the model is validated and tested on independent datasets with unseen samples to verify the prediction. The model is finally assessed on independent datasets with unknown drug sensitivity for prediction of drug response or synergy. This is followed by confirming the predictions *in-vitro* before translating them in the clinic (Adapted from [62]).

Data selection: Due to the possible inconsistencies between different datasets in the public data repositories, data selection constitutes the most challenging part [72]. Each dataset can separately exhibit a reasonable predictive power but using multiple datasets by integrating them to build a prediction model can further increase the classification accuracy [73].

Feature selection: Biological data for various cell lines, PDXs or patients in terms of mutations, copy number variation, single nucleotide polymorphism, epigenetic data, RNA, and protein expression along with drug sensitivity data exist in various

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pharmacogenomic databases. These can be used as the input data types or features to generate a good prediction model [62, 63]. Gene expression data (transcriptomic features) holds the most predictive power, and integrating it with other genetic features only marginally improves the performance of an ML model for drug response prediction [74, 75]. A smaller feature-to-sample ratio provides better prediction capability as it plays an important role in controlling the variances [76]. However, maintaining this ratio is extremely challenging for a large pharmacogenomic dataset as it normally contains tens of thousands of transcriptomic features and only a few hundreds of samples. Dimensionality reduction involves systematically reducing the number of features by incorporating meaningful descriptions. This improves the prediction accuracy by reducing overfitting [77].

Data normalization: Since values present in the different datasets have been generated from numerous laboratories using different techniques, the range of raw data values varies widely. Thus, combining features by integrating data from various datasets involves the use of feature scaling, a normalization technique that functions to obtain a common scale by changing values of numeric columns in the dataset [62].

## Training the model

An important objective of model training is to generate a generalizable model that can be applied to data beyond the one used for building the model. Overfitting is said to have occurred or the model is said to have 'over-fitted' the data if this objective is not achieved. Multiple factors govern the occurrence of overfitting that include the amount and diversity of the training data [78].

Machine learning algorithms: Different ML algorithms are available to train the model. A hypothetical function with decision variables is built using a training dataset with known outcomes, that can be used for the prediction of unknown samples using supervised learning algorithms. These algorithms have been widely tested for predicting treatment outcomes. Moreover, classification models are also generally built using supervised learning algorithms [62].

Deep learning is a type of supervised learning algorithm that learns from tens of thousands of data points. During training, the deep neural network (DNN) algorithm processes the raw input data through its hidden layers until it reaches the output layer. Some nodes in the hidden layers can be randomly dropped out and thus not used for processing the incoming data from the previous layers. The addition of such random dropout layers along with feature selection that reduces the feature-to-sample ratio aids in reducing the problem of overfitting. Deep learning models can thus learn important complex features from a large dataset and suppress irrelevant variations by amplifying only the important aspects. This algorithm can also

automatically classify the problem as regression or classification upon being fed with suitable input data [79].

Different ML algorithms can be employed for both monotherapy and combination therapy predictions, where the outcome is either regression or a classification variable. Regression or a continuous outcome includes predicting continuous variables such as drug response in the form of IC50 values, whereas classification or categorical outcome involves a binary output for prediction in terms of presence or absence of drug sensitivity or synergy.

In practice, several different computational models employing ML algorithms are trained, compared, and only those models are selected for subsequent independent testing that exhibit the most promising predictive performance. Different statistical indicators are chosen depending on the aim of the prediction task for measuring the predictive performance of the model [63]. While correlations, root mean squared errors, and coefficient of determination  are commonly used as the measures of error in regression models, accuracy and precision values serve as the standard indicators for classification/ categorical models [63, 80]. However, the predictive capability of the model is constrained by variable factors that include the type of cancer investigated, size of the training dataset, selection of drugs and the choice of machine learning algorithm used for modeling [81].


## Testing the model

Once the prediction model is built and trained using a ML algorithm, it is then used for estimating the predictive performance. This is achieved by model cross-validation, where the entire dataset that is available for training is divided into two separate subsets. One of these data subsets is used for actual training of the model, while the other is used for 'validating' the trained model. The latter dataset is thus referred to as the validation, evaluation, or test datasets, and it is completely different from the independent dataset(s) used for testing the model. After training and validation of the model is complete, it is then applied on independent datasets to verify that it can accurately predict responses on unseen samples [63].


## Assessing the model for drug response prediction on an independent dataset with unknown drug sensitivity

After training, validating, and testing the model, it needs to be assessed in a pre-clinical setting first for it to be translated clinically. Foremost, this involves predicting drug response for independent datasets such as the cell lines, PDXs, and patient samples with unknown drug sensitivity. The results from such predictions can then be confirmed from experiments *in vitro* using cell lines, and *in vivo* and *ex*

*vivo* using PDX models and patient materials. The outcomes from such model assessments might thus ultimately aid in clinical trials.

# Advantages and Challenges

## Advantages

Computational prediction of drug responses in cancer research can serve as *in silico* drug screening tools that can significantly contribute to preclinical research. This might prove as a very useful strategy in terms of prioritizing the candidate compounds, that might ultimately aid in efficiently planning and designing experiments, thereby reducing the time and costs [82, 83].

## Challenges/ Limitations

Significant research challenges are involved in predicting drug responses for cancer research. These include a biological challenge, a data challenge, and a technical challenge. Cancer is a highly complex, heterogenous, and a multi-factorial disease that contributes to the biological challenge. The volume, noise and the heterogeneity of the potentially useful available datasets add to the complexity of the data challenge. To comprehend such a large amount of data, the need for integrating it from multiple sources increases using proper harmonization and normalization techniques that further serve as a technical challenge [63].

Currently, data from cell lines are used to develop most of the ML models that are robust, easy to generate, and useful for the generation of hypotheses. Nonetheless, there is a limitation on the collection of cell lines from all cancer types in the public databases, thereby making it difficult to generate cancer type-specific models [63]. Moreover, cell line data cannot capture the effects of microenvironmental contributions that assist in tumor growth [62]. This can be overcome by using tumor organoids, that can probably mimic the tumor environment [84]. However, there is not much pharmacological data available from the tumor organoids. Besides, the use of patient data that is more disease-relevant should supplement cell line data. A lot of primary patient samples will be required to generate a large-scale pharmacogenomic data which is extremely difficult [62]. PDXs can instead be used to overcome this challenge with the recent development of PDX repositories. Thus, there is an increased need from various scientists to openly share patient-derived data from various clinical trials encompassing different cancers, that includes the patient's drug response statistics and their omic profiles to perform clinically relevant model validations.

The compilation of the mutational landscape, copy number variations, and promoter methylation altogether determine changes in the gene expression, that can potentially reflect most of the cellular processes. However, it would be extremely time-consuming and expensive to determine all the above factors for each patient that comprises the baseline gene expression data [62]. Moreover, it would be actually interesting to develop predictive models with the use of drug-induced perturbation data that have only been used in a few studies proving to be very useful for feature selection [85, 86].

Despite enormous challenges, the development of predictive models for monotherapy and combination therapies holds great promise for precision medicine to be used in the near future.

# BCL2 family proteins

## Introduction

Apoptosis is a genetically programmed process of eliminating unwanted, infected, and damaged cells [87]. This essential biological process is involved in regulating tissue homeostasis and immunity, thereby contributing to the development of organisms. Apoptosis is mediated by two major pathways; intrinsic and extrinsic. The intrinsic pathway, also known as the mitochondrial apoptotic pathway is triggered by various cellular stresses and is regulated by BCL2 family proteins. The extrinsic pathway, also known as the death receptor pathway is mediated by the binding of specific ligands to death receptors present on the cell surface. A substitute pathway for killing cells involves the participation of perforin, granzyme A and granzyme B from cytotoxic T-cells and natural killer cells [88]. All these pathways culminate in the activation of cysteine-dependent aspartate-directed proteases, called caspases, that function as the effector enzymes for cleaving various cytoskeletal and nuclear proteins, thereby resulting in highly controlled cell disintegration [89]. This leads to morphological changes characterized by cell shrinkage, nuclear fragmentation, chromatin condensation, membrane blebbing, and formation of apoptotic bodies [90]. Apoptotic bodies consist of nuclear fragments, intact organelles, and cytoplasm [91], that are cleared from the body via phagocytes by phagocytosis without releasing any pro-inflammatory contents [89], thus completing the process of cell death.

## Classification of BCL2 family proteins

The first reports of elevated BCL2 (B-cell lymphoma 2) levels were obtained from follicular B-cell lymphoma (FL) patients harboring t(14;18) chromosomal translocation [92]. Due to this genomic aberration, *BCL2* gene normally present on chromosomal segment 18q21.3 came under the control of the immunoglobulin heavy chain promoter and enhancer element on chromosome 14 leading to its constitutive expression [93]. Thereafter, increased expression of BCL2 has been observed in other hematological malignancies and it has been found to be associated with resistance to chemotherapy [94, 95]. Moreover, elevated BCL2 protein levels contributed to oncogenesis by inhibiting cell death, and not by promoting unlimited

cellular proliferation [96]. This led to the identification of a family of apoptosis regulator proteins from various studies [97], and evading apoptosis was established as one of the hallmarks of cancer [2].
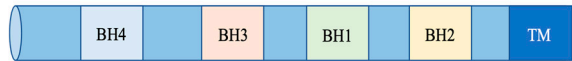
More than 25 BCL2 family members have been identified so far that share sequence homology within conserved regions, termed as the BCL2 homology or BH domains [88]. BCL2 family proteins can be classified based on both their structural and sequence homology in terms of BH domains and their function in the regulation of apoptosis (Figures 6 and 7).

Multidomain anti-apoptotic members such as BCL2, BCL-XL (BCL2L1), BCL-W (BCL2L2), MCL1, and A1 (BCL2A1 or BFL1) possess all the four conserved BH domains (BH1-4). The highly conserved BH4 domain at the N terminus is extremely essential for the anti-apoptotic activity of BCL2 proteins, and it stabilizes BH1-3 domains that form a hydrophobic groove at the C-terminus [88, 94]. This hydrophobic pocket is critical for interaction with the BH3 domains of pro-apoptotic BCL2 family members [89]. Moreover, transmembrane sequences present at the C terminus function to anchor BCL2 family proteins to the intracellular membranes of organelles, mostly the mitochondrial membrane [88]. Indeed, anti-apoptotic members usually reside on the outer mitochondrial membrane (OMM) and preserve its integrity by directly inhibiting the pro-apoptotic proteins. They are also found to be present on the nuclear envelope and the endoplasmic reticulum membrane [98].

The pro-apoptotic family can be further subdivided into two categories structurally and three categories functionally. Structurally, multidomain pro-apoptotic members such as BAX, BAK, and BOK contain three BH domains (BH1-3). Functionally, these proteins are identified as "effectors", that upon activation homo-oligomerize in the mitochondrial membrane and cause the formation of mitochondrial outer membrane permeabilization (MOMP) complex, followed by the release of cytochrome c into the cytoplasm that initiates a cascade of caspase activation [88, 99]. MOMP is considered as the 'point of no return' in the apoptotic pathway [100]. The last structural group of BCL2 family members consists of BH3-only pro-apoptotic proteins that possess only the BH3 domain. This is the minimal death domain required for binding the multidomain anti-apoptotic proteins and pro-apoptotic effectors [101]. This group can be further subdivided into two categories functionally. "Activators" such as BIM, BID, and PUMA can directly activate the "effectors", and even interact with anti-apoptotic proteins. "Sensitizers/De-repressors" such as BAD, HRK, NOXA, BIK, and BMF cannot directly activate the "effectors", but instead can sensitize the cells for apoptosis by interacting with the anti-apoptotic members, thereby releasing the bound "activators" that can now combine with "effectors" ultimately leading to apoptosis [88]. Both the sensitizer and activator BH3-only proteins get activated in response to various cellular stressors, where they serve as the natural antagonists of anti-apoptotic BCL2 family proteins [102].

**Figure 6. Structure of BCL2 family proteins, with respect to sequence homology and domain organization.** Based on the function, BCL2 family proteins can be classified into anti-apoptotic and pro-apoptotic family. The entire BCL2 family consists of functional BCL2 homology (BH) domains that are highly conserved. The anti-apoptotic members contain four BH domains (BH1-4), while the pro-apoptotic members can be sub-divided into multidomain (BH123) effectors and BH3-only sensitizers and activators. Generally, most of the BCL2 proteins, except some of the BH3-only proteins, contain a transmembrane domain (TM) that helps them in anchoring on outer mitochondrial membrane (Adapted from [102] and [103]).

Two models have been proposed for BAX/BAK activation and induction of apoptosis (Figure 8). In the direct activation model, the pro-apoptotic activator proteins such as BIM are sequestered by anti-apoptotic proteins such as BCL2. Since sensitizer proteins such as BAD cannot directly activate BAX and BAK, they exert their pro-apoptotic effect by competitively inhibiting the ability of BCL2 to bind BIM, thereby releasing the activator BIM that is now available to bind and trigger BAX and BAK oligomerization. Thus, BH3-only activator proteins can directly activate the effector proteins, thereby inducing apoptosis [89, 100, 103, 104]. In the indirect activation model, anti-apoptotic BCL2 proteins sequester the active monomeric forms of BAX and BAK, thereby preventing oligomer and pore formation. These effector proteins are displaced from BCL2 in presence of increased expression of BH3-only pro-apoptotic proteins that neutralize BCL2, and this triggers oligomerization of BAX and BAK leading to mitochondrial apoptosis. Thus, this model is also known as the displacement model and it doesn't involve any direct interaction between BH3-only and the effector proteins [89, 103, 105]. Cancer cells where anti-apoptotic BCL2 proteins are pre-occupied by activator proteins are considered as "primed for death" [106]. When exposed to sensitizer proteins or anti-tumor molecules such as BH3 mimetics, such cells can rapidly undergo apoptosis by displacing the activator proteins that can finally activate BAX/BAK [107].
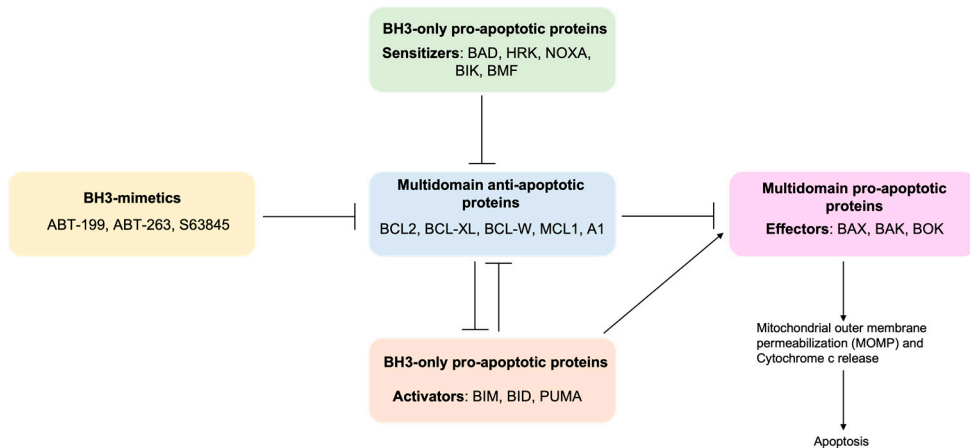
**Figure 7. Interaction among BCL2 family proteins.** Various cellular stressors trigger the intrinsic pathway of apoptosis in the mitochondria, that is strictly regulated by interaction between the pro-apoptotic and anti-apoptotic BCL2 family members. Upon activation, the pro-apoptotic effector proteins cause mitochondrial outer membrane permeabilization (MOMP), followed by the release of Cytochrome c and the activation of executioner caspases ultimately leading to apoptosis. Pro-apoptotic sensitizer proteins cannot directly activate the effector proteins, but instead, they bind to the anti-apoptotic proteins and release bound activators to trigger oligomerization of the effectors. Pro-apoptotic activator proteins directly bind to the effector proteins and activate them. They can also bind to and inhibit anti-apoptotic proteins. In contrast, anti-apoptotic proteins can sequester both the activator and effector proteins, thereby preventing apoptosis. BH3-mimetics mimic the interaction of pro-apoptotic BH3-only proteins, where they bind and inhibit anti-apoptotic proteins from sequestering the activator and effector proteins.

Moreover, anti-apoptotic and BH3-only pro-apoptotic proteins exhibit selective binding and different affinities for each other due to differences in the amino acid sequence of their BH3 domains. BH3-only sensitizers display selective interactions, where BAD favorably binds to the anti-apoptotic proteins BCL2, BCL-XL, and BCL-W, HRK preferentially binds to BCL-XL, and NOXA selectively binds to MCL1 and A1. In contrast, BH3-only activators such as BIM, BID, and PUMA bind to all the anti-apoptotic BCL2 proteins [89, 106].

Apart from the well-studied members of BCL2 family as mentioned above, there are newly identified anti-apoptotic and pro-apoptotic proteins. These include BCL2L10, BCL2L12, BCL2L13 (BCL-RAMBO), BCL2L14, and MAP-1 [94, 108].

The intrinsic apoptosis pathway within mitochondria is thus tightly regulated by the balance and complex network of interactions between anti-apoptotic and pro-apoptotic BCL2 family members. Interruption of this balance can thus lead to a variety of human diseases such as cancers and inflammatory/autoimmune disorders [88].
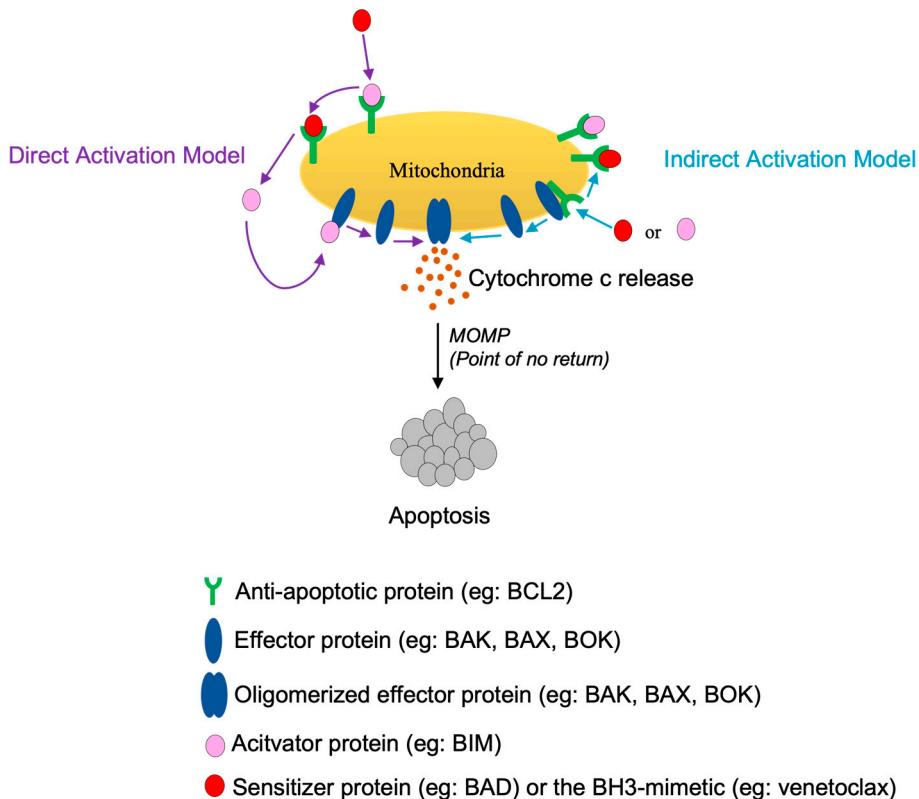
**Figure 8. Models for the activation of BAX/BAK by BH3-only proteins or venetoclax.** BH3-only proteins can activate the effector proteins via two models of activation. This also represents the two modes of action of the BH3-mimetic ABT-199/venetoclax. In the direct activation model, anti-apoptotic protein such as BCL2 sequester the activator protein such as BIM, thereby preventing it from binding and activating the effector proteins, BAX and BAK. Sensitizer proteins such as BAD or even the BH3-mimetic such as venetoclax can displace BIM by competing for binding to BCL2. Free activators can then bind and activate the effector proteins, thereby triggering their oligomerization. In the indirect activation model, the active monomeric effector protein is sequestered by the anti-apoptotic protein such as BCL2, until it is displaced by pro-apoptotic BH3-only proteins or venetoclax binding to BCL2. The effector proteins can now oligomerize leading to MOMP and Cytochrome c release, followed by apoptosis that represents the point of no return.

# Inhibition of BCL2

Many cancers exhibit deregulation of apoptosis, that confers them with a survival advantage over the normal cells. Numerous aberrations of the intrinsic and extrinsic apoptotic pathways identified in hematological malignancies have been associated with prognosis, pathogenesis, and resistance to standard chemotherapeutic drugs [88]. Thus, drugs that can restore apoptosis by targeting components of both the pathways can be used as anti-cancer agents.

Indeed, anti-apoptotic BCL2 is one of the highly upregulated proteins found in many cancers, thereby making it an ideal target for cancer therapy. Very few BCL2 targeting agents have been translated clinically, albeit several of them have been investigated for the past 30 years. Various inhibitors have been developed for targeting BCL2 in cancers using three approaches. The first strategy includes the use of antisense oligonucleotides that can bind to BCL2 mRNA, thereby blocking its translation to BCL2 protein. The last two strategies involve the use of BH3 mimetic peptides and small molecule inhibitors, and they prevent the association of BCL2 with pro-apoptotic proteins [105].

BH3-only pro-apoptotic proteins interact with other subgroups of BCL2 family members via their BH3 domain that represents the canonical site of interaction. As the name suggests, BH3-mimetics have been developed such that they mimic the interaction of BH3-only proteins with specific anti-apoptotic proteins [109]. So far, the use of BH3 mimetic small molecule inhibitors has yielded the most supreme results as compared to antisense oligonucleotide and BH3 mimetic peptide approaches for targeting BCL2 [105]. Moreover, ABT-199 (venetoclax) is the only BH3 mimetic small molecule inhibitor that has been clinically approved by the US Food and Drug Administration (FDA) in 2016 for treating relapsed or refractory CLL patients possessing a 17p deletion who received at least one prior line of therapy [110]. It has also been approved in combination with hypomethylating agents such as low-dose cytarabine, azacitidine, or decitabine for treating newly diagnosed AML patients >75 years old who were unable to receive standard induction therapy [111]. Subsequently, many clinical trials employing ABT-199 as mono and polytherapy have either been initiated or completed for various other cancers.

Venetoclax is a highly selective oral BCL2 antagonist with a high binding affinity for BCL2 ($K_i < 0.01$nM), and low binding affinities for BCL-XL ($K_i = 48$nM), BCL-W ($K_i = 245$nM), and MCL1 ($K_i > 444$nM), while it spares platelets [112]. Since it is a BH3 mimetic, venetoclax can induce apoptosis both by direct and indirect activation of BAX/BAK (Figure 8) [104, 105]. Thus, alterations in the expression of pro-apoptotic and/or anti-apoptotic BCL2 family members can contribute to venetoclax resistance. Four different mechanisms of venetoclax resistance have been identified [89]. These include lack of expression of BCL2 protein, overexpression of other anti-apoptotic proteins, and acquired mutations within the BH3 domain of BCL2 and BAX, thereby abrogating venetoclax binding to BCL2 and BAX activation respectively. Additional mechanisms of venetoclax resistance also exist [104]. Thus, exploring the combinatorial effect of venetoclax with other known anti-cancer agents might be of potential clinical significance.

# Polo-like kinases

## Introduction

Cell cycle is a highly orchestrated process of cell division that consists of G1 (Gap1), S (Synthesis), G2 (Gap2), and M (mitosis) phases. Progression through each of these phases is tightly regulated by various phosphorylation and ubiquitination events that culminate in the production of two daughter cells [113]. The three important serine/threonine kinase families involved in regulating the cell cycle include Aurora kinase (AK), Polo-like kinase (PLK), and Cyclin-dependent kinase (CDK) [114]. The prototypic founding member of the PLK family was first identified as a Polo mutant in *Drosophila melanogaster* over three decades ago. Abnormal spindle poles were induced during mitosis upon knockout of this gene, and hence named "Polo" [115]. PLK is an evolutionarily conserved kinase that is observed in budding yeast (*Saccharomyces cerevisiae* - Cdc5), fission yeast (*Schizosaccharomyces pombe* - Plo1), fruit fly (*Drosophila melanogaster* - polo), amphibian (*Xenopus laevis* - Plxs), and mammals (PLKs) [116].

## PLK family members

Five PLK family members have been identified in humans so far: PLK1, PLK2, PLK3, PLK4, and PLK5, where PLK1 has been most extensively studied [117]. Structurally, all PLKs share the same conserved domain topology with the presence of an amino-terminal kinase domain and a carboxy-terminal polo-box domain (PBD) [118] (Figure 9). The N-terminal kinase domain of PLK1-4 gets activated when its serine/threonine residues are phosphorylated. In contrast, the kinase domain in PLK5 is truncated and is present in an inactive form [119] . The C-terminus of PLK1-3 and PLK5 contain two PBDs that interact with phosphorylated substrates, while PLK4 contains only one PBD [120]. Traditionally, PLK1-4 have been observed to play important roles in genotoxic stress, regulation of cell cycle, and neuron biology. PLK2 and PLK3 are widely expressed in proliferative and non-proliferative tissues, that include the central nervous system and respiratory organs [116]. In contrast, PLK1 and PLK4 are expressed in highly proliferating embryonic tissues and rapidly dividing adult cells like placenta, colon cells, testis, and bone marrow [116, 121]. PLK5 is the only PLK family member that is not involved in

cell cycle regulation [121]. Instead, it plays a role in neuron formation and serves as a tumor suppressor in glioblastoma, thereby being exclusively present in the brain tissue [116, 122]. Thus, PLK family members display structural variation that contributes to their diverse functions in regulating cell cycle, and they also exhibit differential tissue distribution [116, 123].

Since we observed significantly high levels of PLK1 as compared to other family members in our study of T-ALL cell lines, it is discussed in detail below.
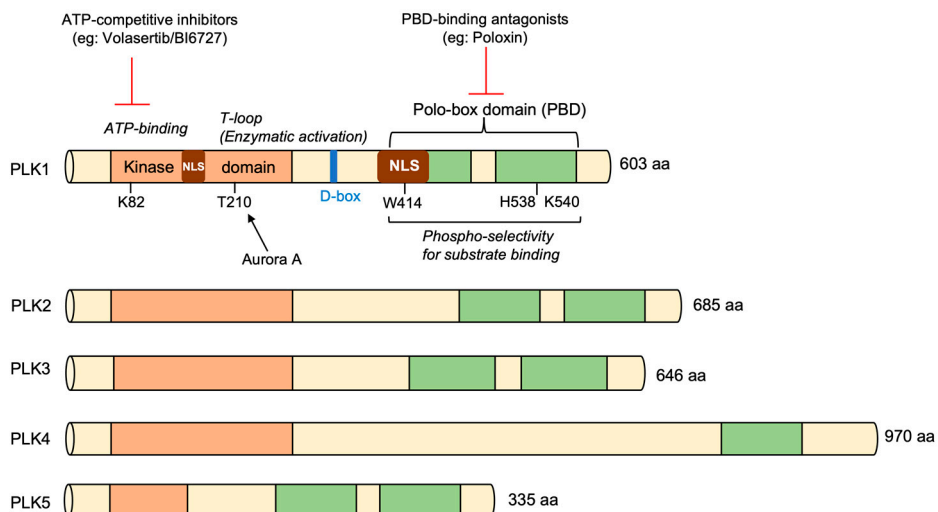
# PLK1

## Structure and activation of PLK1

Two distinct functional domains structurally characterize the PLK1 protein. These include a serine/threonine kinase domain at the N terminus, two PBDs at the C terminus that fold together to form a functional PBD, and an interdomain region in the middle connecting the two domains (Figure 9) [124]. The PBDs of PLK1 are extremely important as they govern its spatial distribution and physical interaction with substrates, thereby controlling its biological activity [125]. Around 622 proteins were identified in the PLK1 interactome that intermingle with PLK1 in a phosphorylation-dependent and mitosis-specific manner [126]. PLK1 also interacts with several other substrates, some of them with non-mitogenic roles [117, 127]. Substrates containing phosphorylated amino acids within their PBD-binding motif (Ser-pSer/pThr-Pro) are recognized and bind to the PBD of PLK1 [118, 128]. While cyclin dependent kinase 1 (CDK1) serves as the priming kinase for most of the substrates (non-self-priming) [127, 128], PLK1 itself can also phosphorylate the PBD-binding motif of some substrates (self-priming) [127]. This facilitates interactions of the PBD of PLK1 with phospho-peptides of the target. Normally, the C-terminal PBD of PLK1 combines with its N-terminal kinase domain through weak intramolecular interactions in the absence of a PBD-substrate interaction. This inhibits the kinase activity of PLK1 by inhibiting the phosphorylation of threonine 210 (T210) residue in the T-loop. However, this autoinhibitory conformation is relieved when the PBD of PLK1 binds to a phosphorylated substrate [118, 129]. PLK1 can now get activated by Aurora kinase A (AuroraA) mediated phosphorylation of its activation T-loop on T210 residue [130, 131]. As a result, it can perform its kinase activity and phosphorylate different sites of the same substrate (direct substrate phosphorylation) or other substrates present nearby (distributive phosphorylation) [118, 127].

Literature also mentions another model of PLK1 activation that is mediated by the co-operated functions of Bora and AuroraA [132]. According to this model, some of the binding partners interact with PLK1 in a phospho-independent or PBD-

independent manner. Aurora borealis or Bora, a co-factor of Aurora kinase A is one such binding partner that associates with the inactive form of PLK1. This association possibly changes the conformation of PLK1 in a way that its kinase domain is relieved from the inhibitory action of PBD. This facilitates AurA mediated phosphorylation of T210 residue in the activation loop of PLK1, thereby activating it. Substrates primed by CDK1 can now bind to the PBD of PLK1, where they can be further phosphorylated by PLK1 during mitosis.

Further, PLK1 consists of two nuclear localization sequences (NLS), one in the kinase domain composed of 13 (134-146) amino acids, and the other in PBD composed of 38 (396-433) amino acids [133, 134]. This indicates that PLK1 shuttles between the nucleus and cytoplasm to interact with appropriate binding partners to support the mitotic function. Additionally, SUMOylation of PLK1 at K492, close to the NLS present in PBD plays an important role in promoting the nuclear import of PLK1 [135].



**Figure 9. Schematic diagram depicting the domain structure of human PLK family.** The N-terminus kinase domains are shown in orange and the C-terminus polo-box domains (PBDs) are shown in green. Only PLK1 contains nuclear localization sequences (NLS), present in the kinase domain and PBD. Key amino acid residues depicted in the kinase domain are essential for ATP binding and enzymatic activation (T-loop), while those mentioned in PBDs play a role in phospho-selectivity for substrate binding. Aurora kinase phosphorylates PLK1 at threonine 210 (T210), thereby activating it. Both the kinase domain and PBDs are separated by a linker domain that comprises of a destruction box (D-box). PLK1 can be targeted with two distinct strategies: ATP-competitive inhibitors such as volasertib/BI6727 inhibit the catalytic activity of PLK1, while PBD-binding antagonists such as poloxin competitively inhibit PBD functions. The total number of amino acids present in each of the PLK family members are indicated on the right (Adapted from [121] and [124]).

## Expression and functions of PLK1

Different stages of cell cycle exhibit significantly different levels of PLK1 expression that correlate with its subcellular localization. In the early period of mitosis, PLK1 continues to gather in the centrosomes of the spindle poles, kinetochores/centromere region of chromosomes, and eventually migrates to the equatorial plate or the spindle midzone as the cell cycle progresses into the middle and late stages of mitosis, and finally gathers in the midbody at the end of mitosis. Thus, PLK1 shows a diminished expression in G1 and S phases, followed by gradual increase in G2 phase, and ultimately peaks in M phase [117, 136]. PLK1 expression sharply declines after completion of cell division due to protein hydrolysis in the late stages of mitosis [137], following which cells enter the G0 (quiescent) state of cell cycle. This skewed bell-shaped curve of PLK1 expression repeats itself in the succeeding loops of cell cycle [117].

Thus, PLK1 plays a very important canonical role in cell division and mediates almost every step in it that includes transition of cell cycle from G2 to M phase or entry into mitosis, maturation of centrosomes, formation and assembly of the bipolar spindle, congression of chromosomes, activation of the anaphase-promoting complex/cyclosome (APC/C), segregation of chromosomes, exit from mitosis, and cytokinesis [138]. Apart from mitosis, PLK1 also performs other important functions such as DNA replication, regulation of microtubule and chromosome dynamics, recovery from DNA damage-induced G2 arrest, and regulation of p53 activity [139].

Various human malignancies express increased levels of PLK1 that often correlates with poor prognosis and increased cellular proliferation [140]. Overexpression of PLK1 in cancer thereby plays important roles in oncogenic transformation, tumor initiation and survival, tumor migration and invasion, induction of epithelial-mesenchymal transition (EMT), and therapeutic resistance. PLK1 overexpression also results in its interconnections with numerous oncogenic signaling pathways, as it regulates the expression and activity of various tumor suppressors and oncogenes by interacting with them [124].

## Inhibition of PLK1

Most of the adult cell types like liver, brain, lung, or kidney are slow dividing under normal conditions where PLK1 cannot be easily detected in them. In contrast, actively proliferating cells possess elevated PLK1 expression [141]. This indicates that PLK1 expression is mainly driven by cell proliferation [142]. Targeting PLK1 can thus serve as an ideal strategy for killing highly proliferative cancer cells, while sparing the normal ones.

Since PLK1 is the key regulator for initiating mitosis, many small-molecule PLK1 antagonists were developed as anti-mitotic and anti-cancer therapeutics [143, 144].

Inhibitors targeting PLK1 belong to three main groups. The first group consists of ATP-competitive inhibitors that target the ATP-binding pocket of the kinase domain at the N-terminus, and these inhibitors affect the kinase activity of PLK1. The second group consists of PBD inhibitors that target the PBD at the C-terminus. The third and the last group of PLK1 inhibitors consists of RNAi-based therapies that result in the depletion of PLK1 protein expression when PLK1 mRNA is targeted. Upon inhibition of PLK1, spindle assembly is disturbed, mitotic checkpoint is activated, and cells get arrested in the pro-metaphase (4n) state of the cell cycle eventually leading to apoptosis. Many PLK1 antagonists have been tested in pre-clinical and clinical studies for acute leukemia, but the ones inhibiting the ATP-binding pocket of PLK1 such as volasertib have been most widely studied [121].

Volasertib (BI6727) efficiently inhibits PLK1 with an IC50 of 0.87nM, thereby inducing G2 arrest and apoptosis in cancer cells. However, it also inhibits PLK2 and PLK3 with IC50 in the low nanomolar range (5 and 56nM respectively) [145]. As per some studies, PLK2 and PLK3 serve as tumor suppressors, and inhibiting these along with PLK1 might reduce the therapeutic benefits of PLK1 inhibition alone [146]. However, volasertib at high concentrations (10μM) showed no inhibitory activity against a panel of more than 50 unrelated kinases [145]. Developing highly selective inhibitors for PLK1 has been difficult due to the high degree of sequence identity observed between all the PLK family members and the high degree of sequence and conformational conservation of the kinase domain of PLK family members with various other kinases [147]. However, despite these limitations, volasertib has a significantly improved safety and pharmacokinetic profile. It is also highly efficacious in multiple preclinical/clinical cancer models [145, 148].

Therapy resistance is the major challenge for any anti-cancer drug development. Combination therapy targeting different cell populations is one of the most efficient ways of inducing significant cell death as compared to monotherapy [121]. An example of one such therapy was the phase I/II study to investigate the combinatorial effects of volasertib with low-dose cytarabine (LDAC) versus LDAC alone in previously untreated AML patients and who were ineligible for receiving intensive treatment [149]. The combinatorial arm of patients showed better response rates and longer survival compared to the LDAC monotherapy arm of patients. Volasertib was thus granted as the "Breakthrough Therapy Status" in AML by US FDA. Later, a phase III POLO-AML-2 trial was thereby initiated, where 666 AML patients aged 65 years and above with no previous treatment and who were ineligible for receiving intensive remission induction therapy were recruited to study the efficacy of volasertib in combination with LDAC [150]. This study got completed in May 2021 and the results are herewith awaited (ClinicalTrials.gov Identifier: NCT01721876).

# The present investigations

## Paper I: Glucocorticoid-resistant B cell acute lymphoblastic leukemia displays receptor tyrosine kinase activation

### Aim

To understand the underlying mechanisms of resistance to dexamethasone in B-ALL.

### Background

Glucocorticoids are essential stress-induced steroid hormones that regulate a variety of physiological processes [151]. Dexamethasone and prednisolone are synthetic analogs of cortisol, and these as well as the endogenous glucocorticoids exert their action by binding to the glucocorticoid receptor (GR) and activating it. GR is encoded by the *NR3C1* (nuclear receptor subfamily 3 group C member 1) gene, a member of the nuclear receptor superfamily, that also serves as a ligand-dependent transcription factor [152]. Glucocorticoids exert strong anti-inflammatory and immune-suppressive functions, due to inhibition of nuclear factor-κβ (NF-κβ) and activator protein-1 (AP-1) regulated gene transcription, and thus are widely used in inflammatory conditions such as autoimmune disorders, and in immune-suppressive treatments following organ transplant [153].

Glucocorticoids were introduced over 50 years ago for the treatment of ALL, and are still widely used along with the chemotherapeutic drugs for the treatment of same and other lymphoid malignancies [154]. These drugs selectively induce apoptosis in ALL cells by promoting glucocorticoid responsive element (GRE) driven transcription of *BCL2L11* gene, that encodes the pro-apoptotic BH3-only protein BIM [155]. However, epigenetic de-regulation of *NR3C1*-induced BIM upregulation was shown to mediate glucocorticoid resistance in ALL. This included increased DNA methylation and reduced chromatin accessibility for binding of GR at the *BCL2L11* locus. These effects were however reversed with the use of DNA

demethylating agents such as 5-azacitidine and decitabine, that enhanced the glucocorticoid response by upregulating *BCL2L11* [156].

Indeed, the glucocorticoid-chemotherapeutic drug regimen in ALL patients has resulted in the long-term survival of 80-90% in children and only 40% in adults, although both the groups exhibit similar complete remission rates [40], thereby implying resistance and relapse of patients. Increased risk of relapse is strongly associated with poor response or failure of ALL cells to achieve effective cytoreduction after 1 week of prednisolone therapy. Moreover, glucocorticoid resistance *in vitro* is associated with an unfavorable prognosis. Thus, primary glucocorticoid resistance is strongly associated with poor prognosis. Further, secondary glucocorticoid resistance predominantly contributes to therapeutic failure, as it is highly prevalent in relapsed ALL [154]. The sensitivity of tumors to glucocorticoid drugs thereby serves as a positive prognostic indicator as they help in achieving clinical remission.

Apart from epigenetic de-regulation, multiple upstream and downstream mechanisms of glucocorticoid resistance have been identified. Some of them include insufficient GR ligand, alterations in the expression and function of GR and its associated proteins such as chaperones, and cross-talk with other cell signaling pathways [157-159]. It is very important to understand the mechanisms of glucocorticoid resistance, as it will aid us in identifying targets and develop novel therapeutic modalities. With this in mind, we aimed at determining the mechanisms of dexamethasone resistance in B-ALL.


## Summary of results and discussion

In order to understand the underlying mechanisms of dexamethasone resistance, we foremost generated dexamethasone-resistant B-ALL cell lines. This was done by exposing three dexamethasone-sensitive B-ALL cell lines (697, NALM-6 and RS4;11) to increasing doses of dexamethasone for a prolonged period until they displayed resistance. The dexamethasone-resistant B-ALL cell line TANOUE was used as a control. The acquisition of dexamethasone resistance was confirmed from their EC50 values (>10µM). All the above-mentioned dexamethasone-resistant B-ALL cell lines were then analyzed by various biochemical assays.

Upon acquiring dexamethasone resistance, we first determined the expression of GR, and found it to be significantly downregulated in NALM-6 and RS4;11 cells. However, GR expression showed a very modest decrease in 697, and it remained unchanged in TANOUE. Dexamethasone resistance was also related to the loss of GR expression in T-ALL [160]. However, the levels of GR expression don't always correlate with sensitivity to dexamethasone [161].

To understand the molecular differences, we performed RNA sequencing for all the dexamethasone-sensitive and -resistant B-ALL cell lines, where only RS4;11

showed a scattered gene expression pattern. However, the other three cell lines displayed almost identical gene expression patterns in their respective sensitive and resistant counterparts. Moreover, dexamethasone-resistant RS4;11 cells displayed enrichment of several kinase and cytokine signaling pathways, implying a switch in their dependency from dexamethasone to kinase-related signaling. Indeed, these cells also showed sensitivity to several receptor tyrosine kinase (RTK) inhibitors. On the contrary, both the sensitive and resistant counterparts of other B-ALL cell lines; 697, NALM-6 and TANOUE displayed similar responses to a panel of 378 kinase inhibitors. Thus, kinase related signaling seemed to play some role in dexamethasone-resistant RS4;11 cells, and so we aimed to identify kinases and RTKs activated in the same. We observed phosphorylation of ERK1/2 and CREB, as well as a strong tyrosine phosphorylation of FLT3 (type III receptor tyrosine kinase (RTK)) and a weak tyrosine phosphorylation of AXL, thereby indicating the presence of a constitutively active RTK signaling in dexamethasone-resistant RS4;11 cells.

Since we observed a constitutive tyrosine phosphorylation of FLT3 in dexamethasone-resistant RS4;11 cells with the help of a human proteome phospho-RTK array, we expected to determine the same in western blots. Indeed, a strong and constitutive tyrosine phosphorylation of FLT3 protein was observed along with the constitutive downstream STAT5 signaling. Moreover, both the FLT3 and STAT5 signaling were inhibited with AC220, a second-generation FLT3 inhibitor.

Out of all the dexamethasone-resistant B-ALL cell lines, RS4;11 possessed FLT3 protein that seemed to contain an internal tandem duplication (*ITD*) mutation based on the type of FLT3 bands seen in western blot. This was also supported by the presence of constitutive tyrosine phosphorylation of FLT3 and STAT5, that usually occurs in presence of *FLT3-ITD* [162, 163]. Moreover, targeted sequencing of around 600 cancer-related genes confirmed the presence of an *ITD* mutation, while Sanger sequencing detected a point mutation (*R845G*) in the *FLT3* of dexamethasone-resistant RS4;11 cells. Thus, these cells carry oncogenic mutants of *FLT3* that respond to FLT3 inhibition. FMS-like tyrosine kinase 3 (FLT3) is widely expressed in the hematopoietic cells. Internal tandem duplication (*ITD*) mutations in the juxtamembrane domain of *FLT3* are the most common genetic alterations in AML; whereas point mutations typically within the kinase-activation loop are less common [164], albeit *R845G* mutation exhibited constitutive activation [165]. Moreover, RS4;11 cell line carries an *MLL-AF4* fusion, and *FLT3* was found to be consistently highly expressed and even frequently mutated in mixed lineage leukemia (*MLL*) rearranged ALLs [166, 167].

Next, we wanted to determine the effect of another second-generation FLT3 inhibitor, crenolanib along with AC220 on the viability of dexamethasone-resistant B-ALL cells *in vitro*. We observed that while only the dexamethasone-resistant RS4;11 cells responded to both the inhibitors, 697 cells did not. This implies that only those dexamethasone-resistant B-ALL cells that rely on constitutive receptor

tyrosine kinase activation due to the presence of *FLT3-ITD* respond to the second-generation FLT3 inhibitors. Moreover, we also detected anti-tumor effects of crenolanib *in vivo* in terms of reducing the tumor weight and size.

Collectively, our data suggested that one of the dexamethasone-resistant B-ALL cell line, RS4;11 displayed constitutive activation of RTK signaling apart from glucocorticoid receptor (GR) downregulation. The fact that these cells responded to second-generation FLT3 inhibitors exhibits their dependency on the activity of oncogenic FLT3 signaling. These results point out different targeted therapies suitable for such dexamethasone-resistant and relapsed B-ALL patients. In particular, if B-ALL patients are screened for *FLT3-ITD* or *FLT3-R845G* mutations, they could possibly be treated with FLT3 inhibitors that are already available clinically.

# Paper II: The Aurora kinase/β-catenin axis contributes to dexamethasone resistance in leukemia

## Aim

To further identify additional mechanisms of dexamethasone resistance in B-ALL.

## Background

From paper I, we saw that long-term dexamethasone treatment with increasing drug concentration induced dexamethasone resistance in B-ALL, and selected for cells containing oncogenic FLT3 mutation. In this paper, we aimed at determining additional mechanisms of dexamethasone resistance, but with a different approach using another B-ALL cell line model system.

Patients often respond to different treatments in a heterogenous manner due to their underlying genomic and proteomic profiles. Thus, to identify a better monotherapy or a combination therapy, great advancements have been made in the bioinformatics area, where computational models are built using various machine learning approaches to better match patients to drugs.

We have already discussed the four basic steps in developing computational models for predicting drug response in chapter III. In this paper, we have used deep learning models for both monotherapy and synergy prediction, with dexamethasone using the same steps. We have also employed transcriptomics, kinase profiling, and drug synergy studies to identify deregulated pathways in dexamethasone-resistant B-ALL cells.

## Summary of results and discussion

Various cell lines respond differently to glucocorticoids. So, we further aimed to determine additional mechanisms of dexamethasone resistance using another B-ALL cell line SUP-B15 in this paper. Foremost, we determined that SUP-B15 cells were highly sensitive to both dexamethasone and prednisolone. So, the micromolar concentrations of these drugs exhibited a significant level of apoptosis after 24h, and more than 90% inhibition in cell viability after 48h. However, there was no substantial apoptosis detected at lower time points i.e., 4h and 6h. To rule out deregulation of gene expression due to apoptosis, we thus chose 6h as the time point for further experiments with glucocorticoids. SUP-B15 cells were then treated with 1µM dexamethasone, 2µM prednisolone, and DMSO for 6h before collecting total RNA, and analyzing it for deregulated genes by Significance Analysis of Microarrays (SAM). We observed that there were many deregulated genes, with similar clusters of the top-listed commonly upregulated genes and similar enrichment of pathways in both the glucocorticoid treated SUP-B15 cells. Thus, short-term exposure to dexamethasone and prednisolone in SUP-B15 cells displayed similar deregulation of gene expression and enrichment of genes associated with epithelial-mesenchymal transition and β-catenin pathways.

Since we observed regulation of kinase activity in dexamethasone-resistant RS4;11 cells in paper I, we hypothesized that short-term dexamethasone exposure might also modulate core cellular signaling by altering the activation of protein kinases. Thus, we measured global kinase activity in SUP-B15 cells treated with 1µM dexamethasone for 6h using peptide substrate-based kinase profiling. We observed that the activity of tyrosine kinases was completely downregulated, while that of several serine/threonine kinases was upregulated. Moreover, we observed a five-fold expression of SPRY1, a member of the sprouty family proteins in dexamethasone and prednisolone-treated cells. Sprouty proteins negatively regulate RTK signaling [168], and might thus be one of the factors behind the downregulation of tyrosine kinase activity.

We had observed enrichment of β-catenin responsive genes in glucocorticoid treated SUP-B15 cells, and thus speculated it to be involved in dexamethasone resistance. β-catenin is a pivotal component of the canonical Wnt signaling pathway. In absence of Wnt signaling, the destruction complex that is composed of Axin, APC, CK1$\alpha$ and GSK-3β phosphorylate β-catenin and target it for β-TrCP mediated ubiquitination and, thereby, proteasomal degradation [169]. However, in presence of Wnt signaling, β-catenin is stabilized and accumulated in the cytoplasm, which then translocates to the nucleus to transcribe β-catenin-dependent genes such as *C-MYC*, *CCND1*, *BIRC5*, and *CDKN1a* that are involved in cell proliferation and survival [170]. Aurora kinases were found to be one of the upregulated protein serine/threonine kinases, and they can directly phosphorylate GSK-3β on Ser9, thereby inactivating it. This results in the accumulation and stabilization of β-

catenin [171]. Indeed, we observed upregulation of β-catenin protein levels in dexamethasone-treated cells. Inactivation of GSK-3β can also be mediated by several other serine/threonine kinases such as p38 [172], ERK [173], AKT [174], and S6K [175]. We then checked the phosphorylation levels of these kinases and observed that while the phosphorylation of p38 and S6K increased with dexamethasone exposure in SUP-B15 cells, the phosphorylation of ERK and AKT decreased. S6K can be activated by the mTORC1 complex that lies downstream of the PI3K/AKT pathway [174]. However, based on our results, it seems that the induction of S6K phosphorylation in presence of dexamethasone occurs independently of AKT activation. For example, S6K activation has been linked to the activation of Aurora kinase [176]. We also checked the phosphorylation of GSK-3β at Ser9 residue and observed that its levels reduced initially and then increased with time in presence of dexamethasone. Moreover, tozasertib, an inhibitor of Aurora kinase along with dexamethasone reduced β-catenin protein levels, implying the requirement of Aurora kinase activity for β-catenin accumulation in dexamethasone-treated cells. Since ERK and AKT displayed constitutive activation in SUP-B15 cells, it might be possible that these kinases possibly regulate the activity of GSK-3β initially by maintaining serine 9 phosphorylation levels. However, as cells get exposed to dexamethasone, Aurora kinase, p38 and S6K might take over the function. Thus, dexamethasone-mediated regulation of multiple signaling pathways stabilizes β-catenin protein levels.

Next, we aimed to verify these findings in ALL patient materials. To achieve this, we would foremost need to know the sensitivity of these samples to dexamethasone. Since TARGET ALL dataset provides gene expression data for ALL patient samples but no inhibitor sensitivity data, we first aimed to predict dexamethasone sensitivity in them. For this, we developed a deep learning model using the 4 steps as described in chapter III:

Selecting datasets and processing the input data: Gene expression data from three datasets were processed and used as the input data to develop a deep learning binary classification model. The first dataset contained SUP-B15 cells that were treated with DMSO, dexamethasone and prednisolone. Deregulated genes from such treatment groups were combined with genes displaying the highest level of variation from the other two datasets; CCLE (917 cell lines) and TARGET ALL (205 patient samples) datasets. RNA expression data for 917 cell lines from different cancers and 205 ALL patient samples are publicly available in the CCLE and TARGET databases respectively. However, the CCLE dataset contains more than 18,000 genes for each sample. A prediction model can be built using such a high number of genes, but it might suffer from poor generalization performance when tested on new data. Therefore, to reduce overfitting, we performed feature selection and attempted to reduce the number of genes in order to have a higher sample-to-feature ratio. This was done by combining gene expression data from the three datasets and only selecting the overlapping genes, which allowed us to construct a 500-gene

signature. Thus, in order to build a deep learning model using 138 cell lines belonging to hematological malignancies from the CCLE database, we used a combined 500-gene signature along with dexamethasone sensitivity data from the PharmacoDB database.

Training the model: We marked cell lines with IC50 < 700nM as dexamethasone-sensitive and those with IC50 > 1000nM as dexamethasone-resistant, and used a binary classification model where the output would be 0 if the cell lines were sensitive and 1 if resistant. The Keras sequential model was used to build our drug sensitivity prediction model.

Testing the model: The deep learning model that we developed was then tested using three sets of samples; 708 CCLE cell lines, and two small ArrayExpress datasets containing 36 and 8 cell lines respectively. When all the three datasets were combined, the model accurately predicted all the sensitive cells, but predicted 684 samples as resistant out of 709. This may be because a larger number of resistant samples were used for testing as compared to the sensitive ones. Overall, despite having 63% negative predictive value (NPV), the model performed well using the three datasets with almost 97% prediction accuracy.

Assessing the model for drug response prediction on an independent dataset with unknown drug sensitivity: Once the deep learning model was developed, we used the same 500 gene signature to predict dexamethasone sensitivity in 205 ALL patient samples from the TARGET database. Around 96 samples were predicted as dexamethasone-sensitive and 109 samples as dexamethasone-resistant. Sensitivity to dexamethasone can predict patient survival [177]. Thus, we compared event-free survival (EFS) between the predicted dexamethasone-sensitive and -resistant ALL groups and observed a significantly reduced EFS for the dexamethasone-resistant group. Almost similar result was obtained for ALL patient samples that were derived from bone marrow but not from the peripheral blood, probably because the latter group contained a smaller patient cohort.

We then compared gene expression between the predicted dexamethasone-sensitive and -resistant ALL patient samples obtained from bone marrow to identify pathway enrichment. We observed that the resistant group displayed almost similar pathway enrichment as the dexamethasone-resistant ALL cell lines, as well as SUP-B15 cells treated with dexamethasone. One such interesting pathway was the one involved in epithelial-mesenchymal transition (EMT). Several EMT-related transcription factors contribute to cell viability, proliferation, stemness and drug resistance in hematological malignancies [178]. It has been shown that dexamethasone favored EMT-related pancreatic cancer progression [179]. Moreover, Aurora kinases have been associated with EMT in breast cancer metastasis [180, 181]. Further, Wnt/β-catenin signaling pathway also associates with EMT [182]. Thus, the activation of Aurora kinases from our kinase profiling data and the increase in β-catenin protein

levels upon dexamethasone treatment relates to the enrichment of EMT markers seen in GSEA.

Several genes including *FLT3* and *SOCS2* were found to be downregulated in predicted dexamethasone-resistant ALL patient samples compared to the sensitive samples. This result was also extrapolated in western blots, as we detected downregulation of FLT3 and SOCS2 protein levels in the intrinsically dexamethasone-resistant cell line TANOUE as compared to the -sensitive cell line SUP-B15. In paper I, we observed that only one dexamethasone-resistant cell line possessed oncogenic FLT3 that resulted in constitutive activation of RTK signaling. Thus, the decrease in FLT3 expression seen in this paper suggests that it might not be a general phenomenon for all the dexamethasone-resistant ALL samples, but instead occurs only in a subset of such cells. SOCS2 acts as the negative regulator of cytokine receptor signaling as it belongs to the suppressor of cytokine signaling family of proteins [183]. Indeed, we observed enrichment of the cytokine and cytokine receptor interaction gene signature in the resistant patient group, which is in line with the decreased *SOCS2* expression.

Next, we attempted to identify drugs that could synergize with dexamethasone, thereby overcoming resistance. We achieved this by developing a deep learning binary classification model that could predict synergy using the same four steps as described before. For this experiment, drug synergy data was obtained from the DrugComb database. Input data consisted of the same 500-gene signature for each of the 29 cell lines, along with 488 chemical features to describe each of the 23 drugs. Thus, a total of 529 combinations were used to develop a binary classification model, where a BLISS score > 3 was considered synergy (defined as 1) and a BLISS score < 2 was considered no synergy (defined as 0). Due to the limited number of cell lines and drug combinations, we divided the entire 529 sample set into 421 training and 108 test samples. Thus, the deep learning model developed was trained using 421 samples, while it was tested on 108 samples, with a moderate accuracy of 82.4%. The model so developed was then used to predict synergy between dexamethasone and 1454 kinase inhibitors in 40 ALL patient samples from the TARGET database. We observed that the model predicted synergy between dexamethasone and 226 inhibitors that inhibit various kinases. Lastly, we validated the model *in vitro* by measuring synergy between dexamethasone and 38 kinase inhibitors in TANOUE cells, where several but not all kinase inhibitors exhibited synergy. Since most of these kinases are involved in β-catenin stabilization, and we observed enrichment of β-catenin upregulation signature in all the GSEAs, we speculated dexamethasone resistance to be linked to upregulation of β-catenin and its transcriptional activity. Indeed, we observed synergy between dexamethasone and several inhibitors targeting β-catenin activity in TANOUE cells.

To summarize, initial exposure of B-ALL cells to dexamethasone induced Aurora kinase activation that could stabilize β-catenin through phosphorylation dependent inactivation of GSK-3β. Aurora kinases can also activate multiple downstream

signaling pathways, such as S6K [184], p38 [185], and JAK2 [186] that can directly or indirectly phosphorylate GSK-3β and inactivate it, thereby stabilizing β-catenin. It has been observed that inhibition of JAK2 activity can suppress β-catenin accumulation [187], while inhibition of AKT or mTOR can reverse glucocorticoid resistance in ALL [188]. Indeed, dexamethasone sensitivity was partially restored when inhibitors targeting the above kinases and β-catenin were combined with dexamethasone in our study. This implies that the Aurora kinase/β-catenin axis mediates dexamethasone resistance in B-ALL via activation of multiple parallel signaling pathways that ultimately result in stabilization of β-catenin.

# Paper III: The mechanism of venetoclax resistance in T-cell acute lymphoblastic leukemia

## Aim

To understand the underlying mechanisms of resistance to the small molecule, BCL2-specific BH3 mimetic venetoclax in T-ALL.

## Background

During the normal development of T cells in the thymus, BCL2 expression is high in the majority of early CD4 and CD8 double-negative thymocytes, while most of them lose expression while differentiating to the CD4 and CD8 double-positive stage. The expression of BCL2 is, however, regained back in mature CD4 or CD8 single-positive thymocytes, which is retained in the peripheral T cells from lymph node, spleen, and peripheral blood. This biphasic expression of BCL2 in the different stages of T cells might thus be important for their survival, by possibly regulating programmed cell death during positive and negative selection [189, 190].

Chonghaile T N. et al., 2014 [191] showed that BCL2 expression also varied similarly in T-ALL cells depending on their maturation stage. The immature double-negative thymocytes in T-ALL showed dependency on BCL2, which shifted to BCL-XL in double-positive and immature single-positive thymocytes, while the mature single-positive thymocytes showed BCL2 dependency again. Thus, ETP-ALL, a malignancy arising from the immature T cells is dependent on BCL2, and is thus sensitive to the BH3 mimetics ABT-199 (Venetoclax) and ABT-263 (Navitoclax). Venetoclax is a BCL2 specific inhibitor [112], while navitoclax binds and inhibits BCL2, BCL-XL and BCL-W [192]. Thus, malignancy arising from the mature double-positive thymocytes (typical T-ALL) shows sensitivity to navitoclax as it is BCL-XL dependent. T-ALL cell lines and primary patient samples also

showed BCL-XL dependence. Thus, T-ALL cells exhibit a differential response to the aforementioned BH3 mimetics, depending on their maturation stage and anti-apoptotic protein dependencies [191].

Navitoclax has shown promising results as a monotherapy in clinical trials for CLL patients. However, its use is limited in the clinic due to on-target thrombocytopenia, as platelets are dependent upon BCL-XL for survival [193]. Venetoclax was thus developed as a BCL2-specific BH3 mimetic, in order to overcome BCL-XL inhibition in megakaryocytes. Indeed, it caused little or no thrombocytopenia by sparing platelets both *in vivo* and *in vitro* in patients with hematological malignancies [112].

Venetoclax served as an effective monotherapy for an ETP-ALL cell line LOUCY both *in vitro* and *in vivo* [191, 194], along with a number of primary human T-ALL patient samples depending on their immunophenotype and BCL2 expression [194]. It was observed that leukemic cells from one such T-ALL patient exhibited an immunophenotypic switch at relapse, showing high BCL2 expression. The therapy-resistant leukemic cells at diagnosis from this patient thus showed sensitivity to venetoclax at relapse *in vitro*, thereby serving ABT-199 as a promising therapeutic drug in refractory T-cell leukemias [194]. In fact, a phase I clinical study is currently ongoing by recruiting pediatric and young adult patients with relapsed or refractory malignancies, including ALL, to evaluate the safety, efficacy, pharmacokinetics, and the dose limiting toxicity (DLT) of venetoclax monotherapy (ClinicalTrials.gov Identifier: NCT03236857).

However, some tumor cells might show resistance to venetoclax monotherapy. Thus, it is extremely important to identify the potential mechanisms of venetoclax resistance, in order to develop effective therapies and also for patient stratification [195]. In this paper, we have focussed our attention on the expression of various BCL2 family members in T-ALL and have also aimed to identify the mechanisms of resistance upon long-term treatment with venetoclax.


## Summary of results and discussion

One of the hallmarks of cancer is to evade apoptosis [2], and it is often mediated by the elevated expression of anti-apoptotic BCL2 family proteins, that can lead to chemotherapy resistance [196]. With this in mind, we first determined the gene expression data of four BCL2 family members; *BCL2, BCL2L1*, *BCL2L2*, and *MCL1* in seven T-ALL patient cohorts. Three of these cohorts also contained patients annotated as ETP-ALL. We observed a differential expression of BCL2 family members in all the cohorts. *MCL1* expression was consistently high in all the cohorts, except one, while *BCL2L2* expression was the lowest. On the contrary, *BCL2* and *BCL2L1* expression were comparatively higher in the majority of cohorts, albeit *BCL2* expression showed a high variation in six out of seven T-ALL patient

cohorts. Next, we thought that since ETP-ALL samples highly depend on *BCL2* for their survival, they would show an elevated *BCL2* expression, which might thereby contribute to the increased variation seen in gene expression. Thus, we removed ETP-ALL patient data from three cohorts that contained some patients labeled as ETP-ALL. By doing so, we observed a slight reduction in variation of *BCL2* gene expression; however, the cohorts still showed a similar pattern of expression of the BCL2 family members as observed when considering the ETP-ALL patients. Thus, *BCL2* expression could be increased in non-ETP-ALL patients as well.

High BCL2 and low BCL-XL or MCL1 expression correlated with venetoclax sensitivity in multiple myeloma both *in vitro* and *in vivo* [197]. In order to determine such predictors of venetoclax response in our T-ALL cell lines, we assessed the expression of the above-mentioned anti-apoptotic BCL2 family members in a panel of 14 T-ALL cell lines at both the mRNA and protein levels. As expected, BCL2L2 expression was low to undetectable, while MCL1 expression was consistently high in most of the T-ALL cell lines. In contrast, BCL2 expression was low in three cell lines, but displayed relatively higher expression in others, while BCL2L1 expression was relatively low in LOUCY and MOLT-16 cells but higher in others. BCL2/MCL1 ratio was almost the same in all cell lines at mRNA levels, but showed variable expression at the protein level. BCL2/BC2L1 ratio was comparatively higher in only LOUCY and MOLT-16 cell lines, while others displayed almost equal levels. Thus, we hypothesized that these two cell lines would be sensitive to the BCL2-specific inhibitor venetoclax. As expected, both LOUCY and MOLT-16 were sensitive to venetoclax. However, ALL-SIL also showed sensitivity to venetoclax although it displayed a lower BCL2/BCL2L1 ratio. Thus, BCL2/BCL2L1 ratio cannot always reliably predict venetoclax sensitivity in T-ALL cells. All the other T-ALL cell lines were resistant to venetoclax. So, high levels of BCL2 and low levels of other BCL2 family members do not always correlate with sensitivity to venetoclax. On the contrary, BCL2L1/MCL1 ratio was high in most of the cell lines, except a few. Since most of the T-ALL cell lines exhibited elevated levels of BCL2L1, we employed navitoclax that targets BCL2, BCL-XL and BCL-W. We observed that those cell lines that displayed increased BCL2L1/MCL1 ratios showed sensitivity to navitoclax and vice-versa, except ALL-SIL, LOUCY and MOLT-16. Thus, BCL2L1/MCL1 ratio also cannot always reliably predict navitoclax sensitivity in T-ALL cells. Moreover, we observed a differential response of various T-ALL cell lines to the use of highly specific or multi-targeted BCL2 inhibitors, thereby implying heterogeneity in them.

Since venetoclax monotherapy in different types of leukemia and lymphoma often results in resistance and tumor progression [198], we hypothesized to generate venetoclax resistance in T-ALL cell lines in order to understand the underlying mechanisms of its resistance. This was done by exposing venetoclax-sensitive T-ALL cell lines (MOLT-16, ALL-SIL and LOUCY) to increasing doses of

venetoclax for a prolonged period until they displayed resistance to atleast 5μM venetoclax.

Upregulation of anti-apoptotic BCL2 family proteins is one of the main determinants of venetoclax resistance in multiple hematological malignancies [198]. Thus, to get a comprehensive overview into the nature of venetoclax resistant mechanisms, relative mRNA expression of BCL2, BCL2L1, and MCL1 was determined in resistant cells at increasing venetoclax concentrations. MOLT-16 and LOUCY displayed a similar pattern of expression in venetoclax resistant cells. In contrast, ALL-SIL showed a variable expression pattern, where BCL2L1 expression was increased right from the lower concentration of venetoclax and was sustained till 10μM concentration. BCL2/BCL2L1 ratio increased in MOLT-16 resistant cells, while it decreased in ALL-SIL and LOUCY resistant cells. On the other hand, BCL2L1/MCL1 ratio increased in ALL-SIL resistant cells.

Further, venetoclax-resistant MOLT-16, ALL-SIL and LOUCY cell lines were analyzed by RNA sequencing. We observed that MOLT-16 and ALL-SIL resistant cells were quite different from their sensitive counterparts as they clustered differently. However, LOUCY resistant cells clustered with all the sensitive cell lines. We also observed cell line-specific transcriptional regulation, along with differential regulation of BCL2 family gene expression in all the three venetoclax-resistant cells. A lower BCL2:Bim expression ratio was found to be associated with venetoclax sensitivity in primary follicular lymphoma patient samples [199]. On similar lines, we observed a downregulated expression of one or two pro-apoptotic genes in MOLT-16 and ALL-SIL venetoclax resistant cells. Activation of several signaling pathways regulates the expression of both the pro- and anti-apoptotic BCL2 family members, thereby contributing to venetoclax resistance [198, 200]. We observed different cytokine signaling pathways that were enriched in all the three venetoclax-resistant cells, along with enrichment of different sets of cancer stem cell markers. In line with this, we also observed strong phosphorylation of STAT5 in venetoclax-resistant cells. In T-ALL cells, STAT5 mediated downregulation of BCL6 and upregulation of PIM1 in response to IL-7 stimulation, thereby contributing to cell cycle progression and proliferation [201]. Thus, strong phosphorylation of STAT5 seen in our venetoclax-resistant cells probably activates downstream signaling pathways involved in cell proliferation and survival.

Apart from changes in the expression of BCL2 family members, there are some additional mechanisms of venetoclax resistance. Considering the function of BCL2 in preventing apoptosis, it is usually overexpressed in a variety of human neoplasms via diverse mechanisms such as chromosomal translocations, gene amplifications and deletions [202]. Moreover, mutations in BCL2, especially in the BH3 binding groove, might confer resistance to venetoclax. Indeed, a mutation in the BH3 binding groove of BCL2 (F104L) was discovered in a venetoclax-resistant lymphoma cell line, that could potentially interfere with binding of venetoclax [203]. Two similar mutations (F101C and F101L) identified in a mouse B-cell

lymphoma cell line also contributed to venetoclax resistance [204]. In another study, a novel recurrent G101V mutation in BCL2 was identified in patients with progressive CLL that confers venetoclax resistance [205]. Further, BCL2 phosphorylation has been found to be associated with venetoclax resistance, as the pro-apoptotic proteins Bim and Bax could not be displaced from phosphorylated BCL2 by venetoclax, thereby blocking apoptosis through the intrinsic mitochondrial pathway [206].

Since venetoclax-resistant LOUCY cells clustered with the sensitive cells, and they didn't show any change in the gene expression of anti-apoptotic and pro-apoptotic BCL2 family members, they might probably be harboring mutations or post-translational modifications in BCL2 or related family members that might confer resistance to venetoclax. Thus, apart from determining the expression levels of BCL2, identifying any genetic abnormalities and post-translational modifications in it might be another area to explore in our venetoclax resistant cells, that might possibly contribute to resistance.

To summarize, we have identified numerous non-universal changes in all the venetoclax-resistant cell lines tested. This is possibly due to the underlying inherent genetics of each T-ALL cell line; that further illustrates the complexity and heterogeneity of potential venetoclax resistance mechanisms.

# Paper IV: BCL2 inhibition synergizes with PLK1 inhibition in T-cell acute lymphoblastic leukemia

## Aim

To identify a possible therapy in combination with venetoclax using T-ALL patient samples, cell lines and patient-derived xenografts (PDXs), and also understand the underlying mechanisms of synergy.

## Background

Venetoclax may serve as an effective anti-leukemic monotherapy, albeit the possibility of therapeutic resistance may limit its use as a single agent [207]. Thus, combining venetoclax with chemotherapeutic drugs or other targeted therapies might potentially increase the chemosensitivity of leukemic cells, protect against the emergence of therapeutic resistance, and decrease the dose-dependent side-effects of chemotherapy [194]. Indeed, notable clinical activity has been observed in relapsed or refractory T-ALL patients treated with venetoclax in combination with chemotherapy [208], nelarabine [209], decitabine [210], and bortezomib [211].

Moreover, venetoclax in combination with chemotherapy is currently being tested in a phase 1b clinical study as a frontline therapy for older patients and patients with relapsed/refractory ALL (ClinicalTrials.gov Identifier: NCT03319901), where changes in the expression of both pro- and anti-apoptotic BCL2 family proteins will be monitored throughout.

Strong synergism was observed between ABT-199 and cytarabine in an early immature LOUCY cell line, but not in the more-differentiated T-ALL cell lines [212]. However, synergistic effects between ABT-199 and other chemotherapeutic drugs such as doxorubicin, L-asparaginase, and dexamethasone were observed not only in LOUCY, but also in more mature T-ALL cell lines such as JURKAT and ALL-SIL [194]. Thus, the heterogeneous response of T-ALL cell lines to different combinations of chemotherapy with venetoclax points to the need of finding a more homogenous and an effective targeted therapy in combination with venetoclax.

Moreover, targeting other anti-apoptotic proteins such as BCL-XL and MCL1 in combination with BCL2 might also seem as an attractive therapeutic option, since upregulation of these proteins might be responsible for venetoclax resistance. In fact, a phase I dose-escalation study was completed early this year for children ($\geq 4$ years old) and adults with relapsed/refractory T-ALL, B-ALL or lymphoblastic lymphoma, to evaluate the safety, efficacy, and pharmacokinetics of venetoclax in combination with navitoclax and chemotherapy (ClinicalTrials.gov Identifier: NCT03181126). This treatment combination displayed marked response rates and a well-tolerated safety profile in most of the patients who had failed multiple therapies, including immunotherapies, targeted agents, and stem cell transplants. However, directly inhibiting more than one anti-apoptotic protein comes with particular side effects, such as neutropenia and thrombocytopenia. These cytopenias occurred quite frequently, suggesting that intermittent dosing along with growth factor support might be needed when both the BH3 mimetics (navitoclax and venetoclax) are used in combination with chemotherapy [213].

Thus, understanding the mechanisms of venetoclax resistance, along with identifying biomarkers that can predict venetoclax sensitivity are extremely important in order to identify optimal combination partners with venetoclax, other than chemotherapeutic agents and BH3 mimetics [198].


## Summary of results and discussion

Since we determined the mechanisms of venetoclax resistance using T-ALL cell lines in the previous paper, we foremost aimed to determine the same using T-ALL patient samples in this paper. To identify the mechanisms of venetoclax resistance in these samples, we would primarily need to know their sensitivity towards venetoclax. However, since there is no information on the BCL2 inhibitor sensitivity data for the patient samples, we first aimed to predict venetoclax sensitivity in them

using a deep learning model as described in paper II. This helped us in bifurcating patients from two T-ALL patient datasets (GSE28703 and GSE78132) as venetoclax-sensitive and venetoclax-resistant with 91.6% prediction accuracy.

Since we observed that BCL2/BCL2L1 ratio predicted venetoclax sensitivity in two out of three venetoclax-sensitive T-ALL cell lines in paper III, we also aimed to identify the same in the aforementioned T-ALL patient cohorts. We observed that T-ALL patients predicted as venetoclax-sensitive displayed a higher BCL2/BCL2L1 ratio in the GSE28703 cohort as compared to its resistant counterpart. On the contrary, there was no difference seen in the other cohort. Thus, similar to the T-ALL cell lines, BCL2/BCL2L1 ratio cannot always predict venetoclax sensitivity in T-ALL patient samples too. We then wanted to identify pathways augmented in the patient samples that are predicted as venetoclax-resistant from both the cohorts, and observed that the Aurora kinase and PLK1 pathways were highly enriched. Since we observed enrichment of kinase pathways here, and most of the T-ALL cell lines showed sensitivity to navitoclax in the previous paper, we wanted to identify a kinase inhibitor that might further enhance the effect of navitoclax. We observed that out of a panel of 378 kinase inhibitors, those targeting PLK1 were mostly effective. Thus, PLK1 was identified as the kinase that could be targeted in combination with the BCL2 inhibitor in T-ALL.

PLK1 was found to be abundantly expressed in a variety of human leukemia cell lines, as well as in the leukemic cells from AML and ALL patients as compared to the mononuclear cells isolated from the peripheral blood and bone marrow of healthy volunteers [214]. We thus aimed to determine the mRNA levels of all the five PLK family members in our panel of 14 T-ALL cell lines. We observed that while PLK1 expression was relatively much higher in all the T-ALL cell lines as compared to PLK4, expression of PLK2, PLK3 and PLK5 was low to undetectable. Moreover, like the mRNA levels, PLK1 protein levels were also heterogenous in T-ALL cell lines.

PLK1 levels are strongly correlated with the aggressiveness and prognosis in many cancers, thereby serving as a novel diagnostic marker, that when inhibited could function as an important anti-cancer therapy [140]. Indeed, anti-mitotic drugs have proven to be effective therapeutic agents in various cancer types that interfere with the process of cell division, and these include those targeting PLK1 as well [215, 216]. Moreover, PLK1 inhibition has been shown to enhance the sensitivity of cancer radiotherapy, and also overcome drug resistance in cancer chemotherapy [117]. We selected volasertib as the PLK1 inhibitor to be used in our study, since it has been granted as the "Breakthrough Therapy Status" in AML by US FDA. Almost all the T-ALL cell lines used in our study showed sensitivity to volasertib, except TALL-1. Additionally, T-ALL PDXs were also heterogeneously sensitive. PLK1 inhibitor volasertib has the potential to inhibit PLK2 and PLK3 with comparatively lower potencies, but it possesses no activity against PLK4 [148]. Thus, altogether, volasertib seems to specifically inhibit PLK1 in our study.

Since PLK1 inhibition worked in combination with navitoclax, we hypothesized that PLK1 inhibition might probably also work in combination with venetoclax, thereby overcoming venetoclax resistance. The combination of volasertib and venetoclax served as an effective therapeutic strategy for *MYC*-overexpressing cancers, such as small cell lung carcinomas and neuroblastomas [217], as well as double-hit lymphomas [218]. Indeed, we observed synergy between a range of concentrations of venetoclax and volasertib in some T-ALL cell lines. Interestingly, high synergism was also observed in two T-ALL PDXs (T-ALL-27 and T-ALL-44) that were relatively less sensitive to volasertib. On the contrary, PDXs (T-ALL-42 and T-ALL-46) that were highly sensitive to volasertib exhibited no synergistic effect. The synergistic induction of apoptosis was also observed in some T-ALL cell lines, which correlated with the increased sub-G1 phase in cell cycle analysis for those cells. Moreover, NSG mice that were engrafted with the luciferase-expressing DND-41 cells and treated with the combination of venetoclax and volasertib, exhibited reduced tumor burden and improved overall survival as compared to the other treatment groups of mice.

Next, we wanted to identify the underlying mechanisms of synergy between venetoclax and volasertib. For this, we foremost determined whether PLK1 transcriptionally regulates any BCL2 family members. Four T-ALL cell lines were treated with the PLK1 inhibitor volasertib for 24 hours, followed by mRNA quantification of 21 BCL2 family genes. We observed that the expression of some genes did not significantly change upon exposure to volasertib. However, some genes showed a significant upregulation, including *BCL2L13 (BCL-Rambo)* and *PMAIP1 (NOXA)*. Upon exposure to volasertib, this result was also extrapolated at both the mRNA and protein levels in two PDXs; T-ALL-27 and T-ALL-44, that showed synergy between venetoclax and volasertib. BCL2L13 can perform both pro- and anti-apoptotic cellular functions depending on different conditions. As a pro-apoptotic protein, BCL2L13 interacts with the mitochondrial permeability transition pore (MPTP) and induces the release of cytochrome C into the cytosol, resulting in the activation of caspase cascade [219]. PMAIP1 has been shown to enhance venetoclax sensitivity in AML cells by sequestering MCL1 [220, 221]. It has also been shown to enhance bortezomib sensitivity in neuroblastoma by neutralizing BCL-XL [222]. We also observed enrichment of several metabolic and biosynthesis pathways in T-ALL-44, suggesting that venetoclax/volasertib-induced drug synergy probably involves metabolic regulation. Higher accumulation of certain metabolites which are components of similar metabolic pathways was observed in T-ALL-42 and T-ALL-46 as compared to T-ALL-44. One such metabolite identified was the arachidonic acid. On the other hand, higher arachidonic acid metabolism was observed at the transcriptional level in T-ALL-44, which correlates with its reduced accumulation. Linoleic acid can be enzymatically converted to arachidonic acid, and further metabolized to produce series 2 prostaglandins [223], that via activation of multiple signaling pathways can lead to phosphorylation-dependent inactivation of GSK-3β, thereby stabilizing β-catenin

[224, 225]. Indeed, both the PDXs T-ALL-27 and T-ALL-44 displayed higher GSK-3β-Ser9 phosphorylation, thereby stabilizing β-catenin.

Transcription of BCL2L13 and PMAIP1 is regulated by unphosphorylated Forkhead box-O (FOXO) transcription factors [226, 227]. β-catenin acts as the partner of FOXOs in the nucleus [228]. However, PLK1 can phosphorylate FOXOs and induce their nuclear exclusion [229, 230], thereby deactivating BCL2L13 and PMAIP1 transcription. PLK1 inhibition by volasertib thus induced BCL2L13 and PMAIP1 expression in PDXs T-ALL-27 and T-ALL-44 that displayed higher synergy. Moreover, these PDXs expressed high β-catenin levels that further supports the hypothesis that PLK1 inhibitor-mediated stabilization of FOXOs in co-operation with β-catenin drives the expression of BCL2L13 and PMAIP1. Besides, PLK1 inhibition by volasertib did not alter GSK-3β-Ser9 phosphorylation, and thus β-catenin expression. Therefore, PLK1 inhibition by volasertib co-operates with BCL2 inhibition by venetoclax in T-ALL, with the help of sustained β-catenin levels.

We also observed enrichment of oxidative phosphorylation (OXPHOS) in T-ALL-42 and T-ALL-46, two PDXs that displayed higher sensitivity to volasertib. This is in line with the study of AML patients that exhibited increased sensitivity to the PLK1 inhibitor onvansertib in association with upregulated OXPHOS levels at baseline [231]. Moreover, OXPHOS is linked to drug resistance in cancer cells, that can be reversed using anti-cancer drugs combined with those targeting OXPHOS [232]. Human leukemia stem cells (LSCs) of AML overexpress BCL2 [233], that might promote their survival in response to chemotherapy and irradiation, thereby contributing to drug resistance [234]. Metabolically, BCL2 mediates oxidative phosphorylation that is essential for the energy homeostasis of LSCs. LSCs thus exhibit a selective dependency on OXPHOS for their survival, as its inhibition does not lead to upregulation of glycolytic pathways. Moreover, targeting BCL2 with BH3 mimetics selectively eradicated quiescent LSCs by inhibiting OXPHOS [233]. Likewise, overexpression of BCL2 in leukemia cell lines can influence mitochondrial biogenesis resulting in increased oxidative phosphorylation, while inhibition of BCL2 results in uncoupling of mitochondrial respiration from oxidative phosphorylation [235]. In our study, we observed that T-ALL-42 and T-ALL-46 exhibited enrichment of similar cancer stem cell markers that might be responsible for the enrichment of oxidative phosphorylation, whereas T-ALL-44 showed a different profile. The intrinsic BCL2 protein expression was extremely low in T-ALL-42, while it was comparatively high in T-ALL-46. Apart from BCL2, BCL-XL and MCL1 also mediate mitochondrial respiration and ATP generation [236]. Thus, it might be possible that instead of BCL2, other anti-apoptotic proteins like BCL-XL or MCL1 might probably be involved in the regulation of oxidative phosphorylation in these two PDXs. This was also supported by the fact that no synergism was observed upon inhibiting BCL2 with PLK1. Thus, identifying whether BCL-XL or MCL1 antagonists would be synthetically lethal to T-ALL-42

and T-ALL-46 could be another area to explore. However, synergism was detected when venetoclax was combined with oligomycin, an inhibitor of oxidative phosphorylation in T-ALL-42, suggesting that oxidative phosphorylation was indeed responsible for cell survival of this PDX. In another study, several cellular regulatory processes such as the upregulation of MCL1 and modulation of lymphoid transcription factors as well as cellular energy metabolism pathways like the AMPK/PKA axis and OXPHOS were involved in venetoclax resistance in lymphoid malignancies. A combinatorial drug approach of venetoclax with metabolic modulators helped in overcoming venetoclax resistance [237].

Collectively, our results suggested that synergy between venetoclax and volasertib is mediated through the transcriptional regulation of BCL2 family proteins, along with the regulation of fatty acid metabolism pathways. On the other hand, increased oxidative phosphorylation is probably one of the reason behind no synergism observed in T-ALL, that can be targeted with metabolic inhibitors along with venetoclax.

# Conclusions and future directions

We investigated the underlying mechanisms of therapy resistance in B-ALL and T-ALL, along with the identification of a possible monotherapy or a combination therapy to treat such drug-resistant cells. We also identified potential mechanisms of synergy in such a combination therapy.

In paper I, we generated dexamethasone-resistant B-ALL cells that were analyzed and found to possess *FLT3-ITD* and *FLT3-R845G* mutations. This led to dependency of such B-ALL cells on oncogenic FLT3 signaling, that could be targeted with second-generation FLT3 inhibitors (AC220 and crenolanib). These results have the potential to be translated into the clinic for dexamethasone-resistant B-ALL patients that are screened for the above mutations.

In paper II, we identified additional mechanisms of dexamethasone resistance using various techniques. We used a deep learning binary classification model to predict dexamethasone sensitivity in ALL patients. The activation of Aurora kinases and its various downstream effector kinases stabilizing β-catenin were identified as the mechanisms of resistance in the predicted dexamethasone-resistant ALL patients, as well as in a dexamethasone-sensitive B-ALL cell line exposed to dexamethasone for a short time. However, a combination of dexamethasone and inhibitors targeting various kinases such as Aurora kinases, JAK, mTOR, S6K, and even β-catenin, partially restored dexamethasone sensitivity in a dexamethasone-resistant B-ALL cell line.

In paper III, we identified cell-line-specific mechanisms of venetoclax resistance in T-ALL. Differential expression of BCL2 family members was observed in venetoclax-resistant T-ALL cells, where the classical expression of increased BCL2/BCL2L1 did not always predict venetoclax sensitivity. Moreover, we observed differential expression of BCL2 family genes and cancer stem cell markers, transcriptional regulation, activation of cytokine signaling, along with increased STAT5 phosphorylation as the cell-line specific mechanisms underlying venetoclax resistance. Identifying chromosomal abnormalities and post-translational modifications in BCL2 that might hamper its binding to venetoclax, are other possible mechanisms of venetoclax resistance that could be explored in the future.

In paper IV, we identified a possible therapy (in combination with venetoclax) to overcome the monotherapy resistance identified in paper III. We observed enrichment of Aurora kinase and PLK1 pathways in the predicted venetoclax-resistant T-ALL patient samples. Thus, inhibiting PLK1 via volasertib synergized with BCL2 inhibition via venetoclax in some of the T-ALL cell lines *in-vitro*, T-ALL PDXs samples *ex-vivo*, as well as in a venetoclax-resistant T-ALL cell line *in vivo*. *BCL2L13* and *PMAIP1* genes were found to be induced upon PLK1 inhibition, that through their respective pro-apoptotic functions synergized with BCL2

inhibition in T-ALL cells with the help of sustained β-catenin levels and regulation of fatty acid metabolism pathway. This study used a limited number of T-ALL PDXs and it would be interesting to see the effects of the combined BCL2 and PLK1 inhibition in more samples. Moreover, identifying synergy in T-ALL PDXs *in vivo* would be noteworthy. It would be interesting to see if a knock-down or knock-out of certain components of the respiratory chain would restore the synergy between BCL2 and PLK1 inhibition in certain T-ALL PDXs. Elucidation of whether BCL-XL or MCL1 acts as the driver of oxidative phosphorylation in such PDXs, that upon inhibition using specific antagonists restores synergy, would be another area to explore. Moreover, we would also like to identify the association between PLK1-FOXOs, and FOXOs-β-catenin to further confirm our hypothesis.

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