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By

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STUDY OF THE EFFECT OF HSP90 INHIBITORS IN THE TREATMENT OF T-ALL AND B-ALL, BY SUPPRESSING THE LCK AND LYN SIGNALING PATHWAYS

Thesis presented and defended at the University of Burgundy,
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Abstract

Title: Study of the effect of HSP90 inhibitors in the treatment of T-ALL and B-ALL, by suppressing the LCK and LYN signaling pathways

Key words: *Acute T-cell and B-cell Lymphoblastic Leukemias, HSP90 Inhibitors, NVP-BEP800, LCK signaling pathway, LYN signaling pathway.*

Abstract: My PhD work relates to T-cell and B-cell acute lymphoblastic leukemia. This project allowed us to identify a novel therapeutic strategy to treat ALL, by using an HSP90 inhibitor, that suppresses LCK and LYN pathways in T-ALL and B-ALL.
Due to the crucial role of both HSP90 and SRC kinases in tumor development, several molecules that inhibit SRC or HSP90 have been developed in order to block cancer cell proliferation.

Our work showed that HSP90 is overexpressed in ALL cells compared to resting B and T cells. Moreover, we showed that HSP90 overexpression is accompanied by an overexpression of LYN kinase in B-ALL and LCK kinase in T-ALL. we found that HSP90 is an important regulator of SRC kinases(LCK and LYN), which are involved in the intracellular signaling pathways required for the growth and proliferation of T-ALL, B-ALL and other types of leukemic cells. The inhibition of HSP90 by NVP-BEP800 induces dissociation of the aberrant HSP90-LYN complex in B-ALL cells and disrupts the entire BCR signaling pathway. LYN and NFκB lose phosphorylation and become inactive, and the latter leave the nucleus, which leads to inhibition of the survival, growth and maintenance of B-ALL cells. We also showed that treating T-ALL cells ,with NVP-BEP800, leads to the calcium / NFAT pathway inactivation, and LCK dephosphorylation, whereas NFAT1 becomes phosphorylated (inactive) and leaves the nucleus leading to inhibition of survival, growth and maintenance of T-ALL cells. These results were published in Blood Cancer Journal in March 2021.

Résumé

Titre : **Étude de l'effet des inhibiteurs de HSP90 dans le traitement des leucémies lymphoïdes aiguës T et B, en inhibant les voies signalétiques LCK et LYN**

Mots clés : *Leucémies Aiguës Lymphoblastiques T et B, Inhibiteurs d'HSP90, NVP-BEP800, Voie signalétique LCK, Voie signalétique LYN.*

Résumé : Mon travail de thèse porte sur les leucémies aiguës lymphoblastiques T et B. Ce projet nous a permis d'identifier une nouvelle stratégie thérapeutique pour traiter différentes types de LAL, en utilisant un inhibiteur HSP90, qui inhibe les voies signalétiques LCK et LYN importantes dans les LAL-T et LAL-B.

En raison du rôle critique des kinases HSP90 et Src dans la progression tumorale, plusieurs molécules inhibitrices de SRC ou HSP90 ont été développées afin de bloquer la prolifération des cellules cancéreuses.

Nos travaux ont montré que HSP90 est surexprimé dans les cellules LALs par rapport aux cellules B et T contrôles. De plus, nous avons montré que la surexpression de HSP90 s'accompagne d'une surexpression de la LYN kinase dans la LAL-B et de la LCK kinase dans la LAL-T. Nous avons constaté que HSP90 est un régulateur important des kinases SRC (LCK et LYN), qui sont impliquées dans les voies de signalisation intracellulaires nécessaires à la croissance et à la prolifération des cellules LAL-T, LAL-B et d'autres types de cellules leucémiques. L'inhibition de HSP90 par NVP-BEP800 induit la dissociation du complexe aberrant HSP90-LYN dans les cellules LAL-B et perturbe l'ensemble de la voie de signalisation "B-cell receptor (BCR)". LYN et NFκB perdent leur phosphorylation et deviennent inactifs, et NFκB quitte le noyau, ce qui conduit à une inhibition de la survie, de la croissance et du maintien des cellules LAL-B. Nous avons également montré que le traitement des cellules LAL-T, avec NVP-BEP800, conduit à l'inactivation de la voie calcium / NFAT et à la déphosphorylation de LCK, alors que NFAT1 devient phosphorylé (inactif) et quitte le noyau entraînant une inhibition de la survie, de la croissance et du maintien des cellules LAL-T. Ces résultats ont été publiés dans Blood Cancer Journal en mars 2021.

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Table of contents

List of Tables

List of Figures

Publication arising from this work

HSP90 inhibitor NVP-BEP800 affects stability of SRC kinases and growth of T-cell and B-cell acute lymphoblastic leukemia

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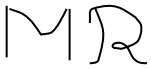
Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Burgundy or any other institution.

Signature: 

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“***Science is what you know, philosophy is what you don’t know*** ”

Bertrand Russel, British philosopher (1872-1970)

Definitions/Abbreviations

A

ABL*: Tyrosine-protein kinase ABL1*

ADP*: adenosine diphosphate*
AF4*:ALL1-fused gene from chromosome 4 protein*
Akt/PKB*: Protein kinase B*
ALL: *Acute lymphoblastic leukemia*
ALL1*: Leukemia, acute lymphocytic, susceptibility to, 1*
AML: *acute myeloid leukemia*
AML1/RUNX1: r*unt related transcription factor1*
An1 B: *anergic B cell*
Ang1*: angiopoietin 1*
APCs: *antigen presenting cells*
ARID5B: *AT rich interaction domain 5B*
ATP: *adenosine triphosphate*

B

BAD: *BCL-2- associated agonist of cell death*
B-ALL: *B-cell acute lymphoblastic leukemia*
BALL-1*: B-cell Acute Lymphoblastic Leukemia-1*
BAX: *BCL-2-associated X protein*
B cell*: B lymphocyte*
BCL2L1*: Bcl-2-like protein 1*
BCR-ABL1*: Philadelphia chromosome*
BMI1 : *B lymphoma Mo-MLV insertion region 1 homolog*
BLNK*: B Cell Linker protein*
BLK*: B-cell lymphocyte kinase*
BIM : *BCL-2 like protein 11*
B-NHL*: B-Non Hodgkin lymphoma*
BRK*: breast tumor kinase*
BTK*: Bruton's tyrosine kinase*

C

CAR-T-cells: *T cells engineered to express chimeric antigen receptors*
CBC: *complete blood count*
Cbl: *ubiquitin ligase*
CD3: *cluster of differentiation 3*
CD4: *cluster of differentiation 4*
CD8*: cluster of differentiation 8*
CD19*: cluster of differentiation 19*
CD28*: cluster of differentiation 28*
CD45*: cluster of differentiation 45*
CD79*: cluster of differentiation 79*
CD81*: cluster of differentiation 81*
CDKN1A: *cyclin-dependent kinase inhibitor1A*
CDK4*: Cyclin-dependent kinase 4*
CDK6*:Cyclin-dependent kinase 6*
c-Kit*: tyrosine-protein kinase KIT*
CLL*: chronic lymphocytic leukemia*
CLP: *common lymphoid progenitors*
CML: *chronic myeloid leukemia*
CMP: c*ommon myeloid progenitors*
C-Myc*: Myc proto-oncogene protein*
C-Myb*: Transcriptional activator Myb*
CNS*: central nervous system*
CSF-1: *colony stimulating factor 1*
CSF: *cerebrospinal spinal fluid*
Csk: *C-terminal SRC Kinase*
CT: *computerized tomography*
CXCL4 :*chemokine (C-X-C Motif) ligand 4*
CXCL12 :*C-X-C chemokine motif 12*

CD22: *cluster of differentiation 22*
CD34: *cluster of differentiation 34*
CEBPE: *CCAAT enhancer binding protein epsilon*
C/EBPα : *CCAAT-enhancer binding protein* α
CTLA-4*: cytotoxic T lymphocyte–associated protein 4*
CVAD: *cyclophosphamide, vincristine, Adriamycin and dexamethasone*

D

DAG*: diacylglycerol*
DCs: *dendritic cells*
DDiT4: *DNA damage-induced transcript 4*
DN: double negative
DP: double positive
DUSP6*: Dual Specificity Phosphatase 6*

E

EAHP: *European association of hematopathology*
EBF1: *early B cell factor 1*
EGFR*: epidermal growth factor receptor*
EGR1: *early growth response protein* 1
EphA2*: ephrin type-A receptor 2*
ERK1/2*: extracellular signal-regulated protein kinase*
ETP-ALL/LBL*: Early T-cell Precursor Acute Lymphoblastic Leukemia*
EPO*:Erythropoietin*
ErP: *erythroid progenitors*
ETS family :E-twenty six
ETV6 : *ETS variant*

F

FAB: *French-American-British classification system*
FISH: *fluorescence in situ hybridization*
Flt3*: Fms-like tyrosine kinase 3*
FO: follicular mature B cells
FOXP1: *forkhead box P1*

G

GADS*: Grb2-related adaptor downstream of Shc*
GATA-2:GATA binding protein 2
GATA-3:GATA binding protein 3
G-CSF: g*ranulocyte colony-stimulating factor*
GMP: *granulocyte-macrophage progenitors*
GRB2*: growth factor receptor-bound protein 2*
GRP-94*: glucose-regulated protein 94*

H

HER2: *human epidermal growth factor receptor 2*
HIF1*: hypoxia-inducible factor 1*
HSCT: *hematopoietic stem cell transplantation*
HSC: *hematopoietic stem cell*
HSP: *heat shock protein*
HSP27*: heat shock protein 27*
HSP60*: heat shock protein 60*
HSP70*: heat shock protein 70*
HSP90*: heat shock protein 90*
HSP110*: heat shock protein 110*
HTLV-1: *human T cell leukemia virus*

I

IgM*: immunoglobulin*
IKZF1: *IKAROS family zinc finger 1*
IL-2 : *interleukin 2*
IL-3: *interleukin 3*
IL-4: *interleukin-4*
IL-5*: interleukin-5*
IL-6: *interleukin 6*
IL-7: *interleukin 7*
IL-10: *interleukin-10*
IL-13*: interleukin-13*
IL-10: *interleukin-10*
IL-13*: interleukin-13*
INFA*: interferon alpha*
INFB*: interferon beta*
iNKT: *invariant natural killer T cells*
IP3*: inositol triphosphate*
IS: *immune synapse*
IR: *ionizing radiation*
IRF-4: *interferon regulatory factor 4*
ITAMs: *immunoreceptor tyrosine-based activation motifs*

J

JAK2: *janus kinase 2*

K

KSHV*: Kaposi Sarcoma-associated herpesvirus*
KS: *Kaposi Sarcoma*

L

LANA: *latency associated nuclear antigen*
LAT: *linker for activation of T cells*
LCK: *lymphocyte cell-specific protein-tyrosine kinase*
LICs*: leukemia-initiating cells*
LINE-1*: Long Interspersed Element1*
LMPP: *lymphoid primed multipotent progenitors*
LYN*: LCK/Yes novel tyrosine kinase*
LP*: lumbar puncture*

M

MAPK: M*itogen-activated protein kinase*
MEK*: Mitogen-activated protein kinase*
MEP: *megakaryocyte-erythroid progenitors*
MRD: *minimal residual disease*
MRI: *magnetic resonance imaging*
mTOR: *mechanistic target of Rapamycin kinase*

MHC: *major histocompatibility complex*

MLL: *mixed-lineage leukemia*

MPN: *Myeloproliferative Neoplasm*
MPP: *multipotent progenitors*

MTSI: *multiple tumor-suppressor gene* 1

MTS2*: multiple tumor-suppressor gene 2*

MZ *: marginal zone B cell*

N

NFAT: *nuclear factor of activated T cells*
NFκB: nuclear *factor-kappa B*
NK: *natural killer*
NCI- high risk category*: National Cancer Institute - high risk category*

NOTCH-1*: Notch homolog 1*
NSG: NOD/SCID/γc−/−
NTD*: N-terminal domain*

P

PAX5: *paired box 5*
PCR: *polymerase chain reaction*

PDX: *patient derived xenografts*

PD-1*: programmed cell death protein 1*
Pi*: inorganic Phosphate*

PIs*: proteasome inhibitors*

PIP2*: phosphatidylinositol biphosphate*
PI3K: p*hosphatidylinositol 3-kinase*
PKC: *protein kinase C*
PLC-γ1*: phospholipase C gamma 1*
PLC-γ2*: Phospholipase C-γ2*
PLZF: *Promyelocytic leukemia zinc finger*

PP2A*: protein phosphatase 2*

PTEN: *phosphatase and tensin homolog*

PUM1 / 2: *pumilio RNA binding family member 1/2)*
PU.1: *purine rich box 1*
p21: *cyclin-dependent kinase inhibitor 1*
p27: *cyclin-dependent kinase inhibitor 1B*

R

RAF*: Rapidly Accelerated Fibrosarcoma*

RAG-1: *recombination-activating gene 1*
RAG-2: *recombination-activating gene 2*

RAS: *rat sarcoma viral oncogene homolog*
RBC: *red blood cell*

S

SCF: s*tem cell factor*

SCL: *stem cell leukemia*
SCT: *stem cell transplantion*

SLP-76*: Lymphocyte cytosolic protein 2*
SP-1: *specificity protein 1*
SPI-1: *spleen focus forming virus proviral integration site 1*

SFKs*: SRC family kinases SFKs*

SH*: SRC homology*
SHP-1: *Protein-Tyrosine Phosphatase SHP-1*
SHP-2: *Protein-Tyrosine Phosphatase 2C*

SRC*: proto-oncogene tyrosine-protein kinase*
Syk: *spleen tyrosine kinase*

T

T-ALL: *T-cell acute lymphocytic leukemia*
TAL-1*: T-cell acute lymphocytic leukemia protein 1*
Tc*: T cell lineage*
T cell*: T lymphocyte*
TCF3: *transcription factor 3*
TCR: *T cell receptor*

TCR ζ chain: *TCR zeta-chain*
TEL/ETV6*: ETS Variant Transcription Factor 6*

TGF-β: t*ransforming growth factor beta*
Th: *T helper cell*
TKIs*: Tyrosine kinase inhibitors*
TLX1: *T cell leukemia homeobox 1*
TPO: *thrombopoietin*
TRAP1*: TNF receptor associated protein-1*
Treg: *regulatory T cell*

U

UV*: ultraviolet*

V

Vav1*: Vav Guanine Nucleotide Exchange Factor 1*
VEGFR1*: Vascular endothelial growth factor receptor 1*
VEGFR2*: Vascular endothelial growth factor receptor 2*

VM*: Vasculogenic mimicry*

W

WBC*: white blood cell*
WHO*: world health organization classification system*

Wnt*: wingless-related integration site*

Z

ZAP-70: *Zeta-chain-associated protein kinase 70*

Others

6-MP*: 6-mercaptopurine*
γ, δ, and ε chains: *gamma, delta and epsilon chains*

β-Catenin: *beta-catenin*
γδ T cells *: Gamma delta T cells*

INTRODUCTION

Leukemia is a group of malignant disorders affecting the blood and blood-forming tissues in the bone marrow, lymphatic system, and spleen. The word leukemia literally means “white blood” because it is a neoplastic proliferation of one type of blood cell, typically a leukocyte or white blood cell. Leukocytosis, an increased white blood cell count, is a normal response to infection, but when leukocytosis becomes chronic or progressively elevates without obvious cause, then it may indicate malignancy. About 5,000 children in Europe are diagnosed with ALL each year **(Roganovic J et al. 2013**). In France, as it is the case in other industrialized countries, acute leukemia (AL) is the most frequent cancer in children aged less than 15 years. Acute lymphoblastic leukemia (ALL) affects mostly children aged between 2 to 5 years old, with 75% of patients under the age of 18, and accounts for 32% of childhood cancers. ALL is a heterogeneous disease that affects T or B lymphocyte precursors in 25% and 75% of cases, respectively. T-ALL and B-ALL development requires multi-step genetic alterations of crucial oncogenes and tumor suppressors *via* different recurrent mechanisms, such as chromosomal translocations, intrachromosomal rearrangements, and mutations in protein-coding genes or enhancer elements, as well as epigenetic abnormalities. These alterations commonly affect survival, proliferation and differentiation of leukemic cells (T-lymphocyte and B-lymphocyte).

Remission rate in children exceeds 80% due to the evolution of the treatments quality. On the other hand, with the current treatment regimens in affected adults, the survival rate is about 60% among those under 55 years of age, and under 30% for patients over 55 years old (**Huguet F et al. 2018***).* Although these regimens induce high rates of complete remission (80 to 90%), an estimated 40% to 50% of adult patients with ALL experience relapse **(Jabbour E et al. 2018 and Kantarjian H et al. 2010).** Thus, the overall cure rate is around 40%, and the five-year overall survival rate is less than 10% with relapsed or refractory ALL **(National Comprehensive Cancer Network, 2019)**. It is therefore necessary to improve the knowledge of this pathology in order to discover new therapeutic strategies that would reduce the intensity of cytotoxic chemotherapy and improve the prognosis of patients after a relapse.

It is with this objective that my thesis work falls. The article “*HSP90 inhibitor NVP-BEP800 affects stability of SRC kinases and growth of T-cell and B-cell acute lymphoblastic leukemia*”, allowed us to identify the interaction between HSP90 and the SRC kinases (LCK, LYN) that are important for the proliferation of leukemic cells. Also it has enabled us to demonstrate that the HSP90 inhibitor (NVP-BEP800), by inhibiting these kinases, decreases the proliferation of leukemic cells and causes them to undergo apoptosis. These results allow us to consider an innovative therapeutic strategy to treat ALL.

This manuscript includes a general introduction which covers all the notions and concepts discussed below: normal hematopoiesis and the regulation of hematopoietic differentiation, different subtypes of leukemia, current treatments for acute lymphoblastic leukemia, the role and importance of chaperone protein HSP90 and SRC family kinase in the progression of leukemia and the HSP90 inhibitors (specifically NVP-BEP800). NVP-BEP800 is a candidate to play a major role in treating acute lymphoblastic leukemia. The results part includes the publication corresponding to the research projects i carried out during my thesis. Finally, the discussion and conclusion parts make it possible to take stock of the work carried out and to discuss the results.

1 Hematopoiesis

Hematopoiesis (/hɪˌmætoʊpɔɪˈiːsɪs, ˈhiːmətoʊ-, ˌhɛmə-/ from Greek αἷμα, "blood" and ποιεῖν "to make", is the formation of blood cellular components. All cellular blood components are derived from hematopoietic stem cells according to body needs. In a healthy adult, approximately one trillion new blood cells are produced daily in order to maintain stable state levels in blood circulation.

Due to the importance of red blood cells in the delivery of oxygen to tissues and the development of vascular channels during embryogenesis, blood formation started at the first week of embryonic life. It occurs first in yolk sac of the embryo from where it migrates to the liver, spleen and lymph node and finally to the bone marrow where the definitive hematopoiesis initiates. Although most of the development of lymphocytes in mammals occurs in the bone marrow, however, maturation and proliferation of some lymphoid cells occurs in the spleen, lymph nodes and thymus (like T cells).

After birth, and during early childhood, hematopoiesis occurs in the red marrow of the bone. In adults, hematopoiesis occurs in the red marrow of the skull, ribs, sternum, clavicles, vertebrae, and pelvis.

1.1 Hematopoietic lineages

Blood is made up of different types of mature cells: lymphocytes, granulocytes (neutrophils, basophils and eosinophils), monocytes, red blood cells and platelets, which are the terminal and functional elements of the two main hematopoietic lineages: the lymphoid lineage and the myeloid

lineage. These cells allow in particular the setting in place of immune responses and oxygen transport. While polymorphonuclear cells have a shelf life of a few hours, platelets are viable for 7-10 days and red blood cells live for around 120 days. In order to be present in constant quantities in the blood of a human (Homo sapiens) adult, hematopoiesis must generate 250 billion red blood cells, 150 billion platelets and 100 billion neutrophils every day.

In the early 1960s, James Till and Ernest McCulloch demonstrated in mice that a single cell in the bone marrow was capable of producing the progenitors of different lineages (**Haas S et al. 2018**). Subsequently, the concept of hematopoietic stem cell (HSC) was defined.

HSCs are able to maintain quiescence, to renew themselves and to differentiate in order to produce all of the hematopoietic lineages (lymphoid and myeloid). This implies a highly regulated process that maintains balance. However, the population of HSC is not homogeneous since not all of them contribute to hematopoiesis in the same way (**Karamitros D et al. 2018**). There is still controversy over the characterization of hierarchies leading to the production of new hematopoietic cells in mammals. Within the framework of the blood hierarchy, several models have been proposed in which four main compartments are described: multipotent HSCs, hematopoietic progenitors, precursors and mature cells.

The classic model (**Figure 1**) describes a differentiation from the most immature cells to the most mature cells (**Orkin S. 2000**). HSCs are first able to generate MPP (multipotent progenitors), which differentiate into CMP (common myeloid progenitors) or CLP (common lymphoid progenitors). The CMP then give other, more differentiated progenitors which are MEP (Megakaryocyte / Erythroid progenitors) and GMP (granulocyte / monocyte progenitors). The GMPs will then generate the monocytic and granulocytic progenitors respectively giving the precursors of monocytes / macrophages and granulocytes. The MEPs form the megakaryocytic and erythroid progenitors respectively producing the precursors of platelets and red cells (**Luis T et al. 2012**).

CLPs differentiate into Pro-B, Pro-T or Pro-NK (natural killer) cells, giving respectively B, T and NK lymphocytes (**Reya T et al. 2001**). This model therefore proposes a strict separation between the myeloid and lymphoid lineages and describes a loss of multipotentiality of the cells during successive differentiations.

Over the past ten years, this model has been modified thanks to the introduction of new techniques such as individual cell analysis systems (**Nimmo R et al. 2015**). The “revised” model presented in **figure 2** results from the identification of a new type of multipotent progenitor called

LMPP (lymphoid primed multipotent progenitors) and the implementation evidence of the ability of HSC to differentiate directly in MEP (**Sanjuan-Pla A et al. 2013**). It suggests that MPPs can differentiate into MEP or LMPP, the latter being capable of giving GMP and CLP. In this second model as in the first, the capacity for self-renewal is only observed in HSC, at the first level of the hierarchy. Currently, the second model is the most recognized. However, the "purity" of the populations which he describes on the one hand and the functional and transcriptional properties which distinguish them on the other hand, are still discussed. Many studies have been published in renowned journals on this subject. Thus, the existence of a continuum between HSC and their immediate descendants is now accepted and calls into question the existence of compartmentalized populations (**Velten L et al. 2017).** In addition, the functional and transcriptional heterogeneities of cells within the populations of LMPP and GMP have recently been demonstrated **(Sanjuan-Pla A et al. 2013** and **Karamitros D et al. 2018).**

Following the study of the gene expression of individualized cells, the authors identified a majority of unipotent progenitors, but also bipotent progenitors and a small proportion of multipotent progenitors, calling into question the uniformity of these populations described in the models of hematopoietic hierarchy. In adults, the majority of HSCs are localized in the bone marrow, where their differentiation takes place.

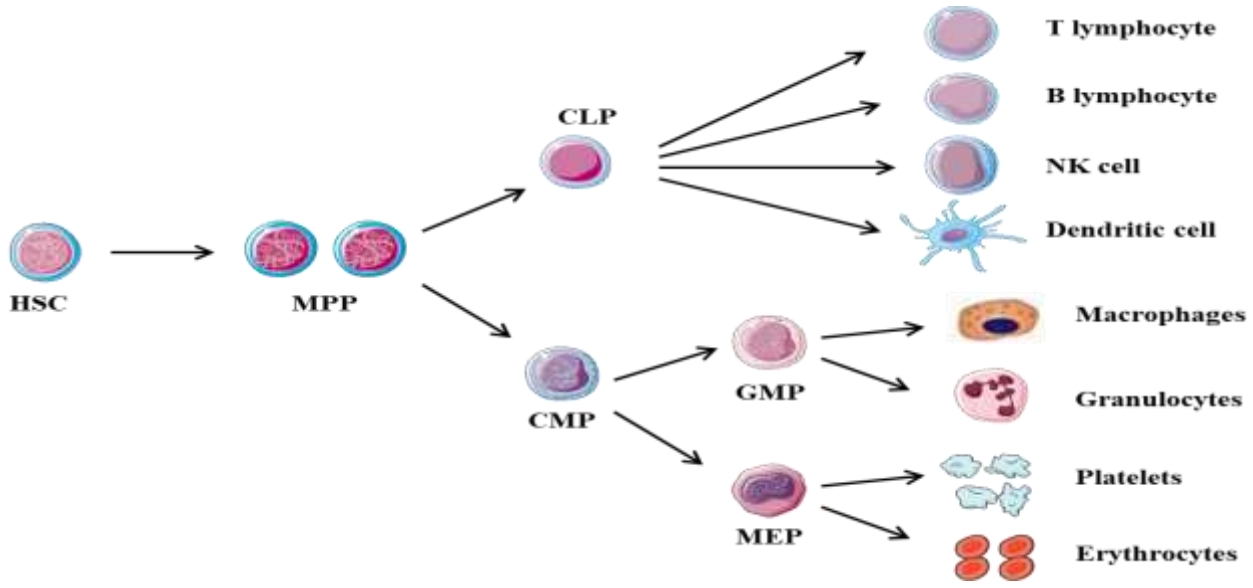

Figure 1: The classical model of hematopoiesis. HSCs reside at the top of the hierarchy; they generate a number of multipotent progenitors (MPPs) which then differentiate into CMP and CLP that develop into myeloid cells and lymphoid cells. B-cell: B lymphocyte, CLP : common lymphoid progenitors, CMP : common myeloid progenitors, GMP : granulocyte/monocyte progenitors, HSC : hematopoietic stem cell, LMPP : lymphoid primed multipotent progenitors, MEP : megakaryocyte/erythroid progenitors, , MPP : multipotent progenitors, T-cell :T lymphocyte ,NK: natural killer cells ,DCs: dendritic cells . Drawn with information from (Luis TC et al. 2012).

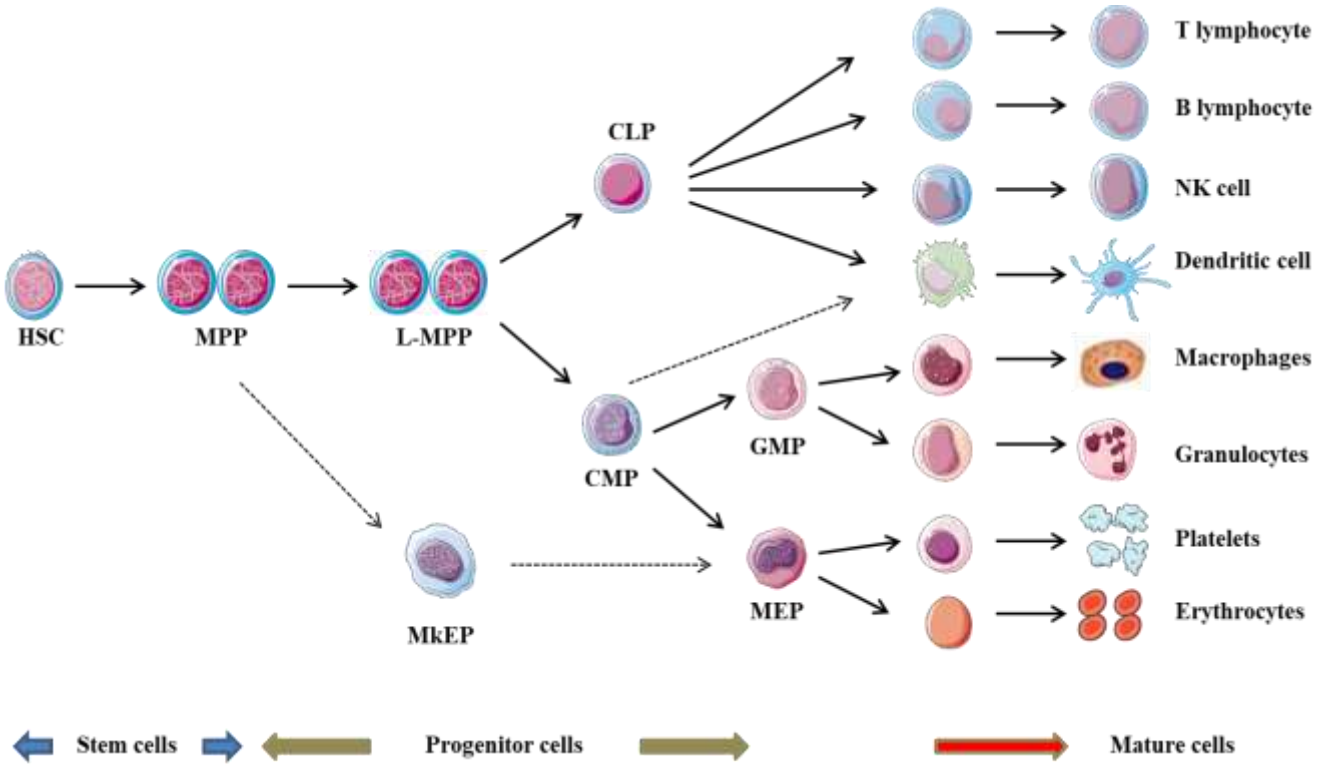

Figure 2: The revised model of hematopoiesis. HSCs reside at the top of the hierarchy, they generate a number of multipotent progenitors (MPPs) which then differentiate into MEP or LMPP, the latter being capable of giving CMP and CLP that develop into myeloid cells and lymphoid cells.B-cell: lymphocyte B, CLP : common lymphoid progenitors, CMP : common myeloid progenitors, GMP : granulocyte/monocyte progenitors, HSC : hematopoietic stem cell, LMPP : lymphoid primed multipotent progenitors, MEP : megakaryocyte/erythroid progenitors, , MPP : multipotent progenitors, T-cell : lymphocyte T,NK: natural killer cells. Drawn with information from (Sanjuan-Pla A et al. 2013 and Karamitros D et al. 2018).

1.2 Hematological microenvironments and signals involved in blood cell differentiation

Within the bone marrow, HSCs benefit from a particular microenvironment in areas called niches. The bone marrow stroma is made up of different types of cells: osteoblasts, fibroblasts, endothelial cells, spinal macrophages, perivascular cells and adipocytes, which first provide physical support for HSCs. These cells also control the fate of HSCs by regulating their quiescence, their self-renewal, their survival, their proliferation and their entry into differentiation thanks to different factors (**Becerra J et al. 2011** and **Anthony B et al. 2014**).

There are two types of hematopoietic niches (**Figure 3**): the endosteal niche, irrigated by arterioles, which keeps HSCs in quiescence and protects them from genotoxic stresses, and the sinusoidal niche where cells are close to the vascular endothelium in spinal sinuses and

proliferate (**Kunisaki Y et al. 2013; Calvi L and Link D. 2015**). The endosteal niche provides a hypoxic environment and contains different types of cells. Osteoblasts produce cytokines involved in maintaining quiescent HSCs such as thrombopoietin (TPO), angiopoietin 1 (Ang1) and CXCL12 (C-X-C chemokine motif 12). Endothelial cells and arteriolar pericytes produce stem cell factor (SCF), CXCL12 and E-selectin. Finally, glial cells are able to secrete TGF-β (transforming growth factor beta) (**Boulais P and Frenette P. 2015**). It has also been shown that this niche is particularly important for the development of lymphoid progenitors (**Ding L and Morrison SJ. 2013; Yu V et al. 2015**). The sinusoidal niche, meanwhile, contains more active HSCs. The megakaryocytes associated with the sinusoidal epithelium control their proliferation and differentiation by producing CXCL4, TGF-β and FGF-1 (fibroblast growth factor 1). There is probably continuous exchange between the endosteal and sinusoidal niches, making it possible to maintain a balance between quiescence and proliferation of HSCs, especially when the hematopoietic niches evolve in response to stress. The key factors being produced by different types of stromal cells, the existence of some functional redundancy is possible **(Wei Q and Frenette P. 2018**).

The fate of HSCs is also controlled by the expression of a transcription program specific to each lineage, involving transcription factors. For example, thanks to mouse models in which it was repressed or overexpressed, Bmi-1 (Bmi1 proto-oncogene, polycomb ring finger1) has been described as being involved in the self-renewal of HSCs (**Park IK et al. 2003** and **Iwama A et al. 2004)**. GATA3 is also involved in their entry into the cycle (**Ku CJ et al. 2012**). c-Myc and c-Myb control the balance between self-renewal and differentiation (**Wilson et al.2004)**. In contrast, EGR1 (early growth response protein 1) keeps HSC quiescent in the niche by inducing the expression of a specific genetic program (**Min I et al. 2008**). MLL (mixed-lineage leukemia) keeps HSC in quiescence but also promotes the proliferation of myeloid lineage progenitors (**Jude, Climer et al. 2007**). Several studies have described the role of TAL1 (T-cell acute lymphocytic leukemia 1) also called SCL (stem cell leukemia) in the survival and self-renewal of HSC (**Reynaud D et al. 2005; Brunet de la Grange P et al. 2006; Souroullas GP et al. 2009** and **Lacombe J et al. 2010**). This transcription factor works by controlling the expression of many target genes, including DDiT4 (DNA damage-induced transcript 4), an inhibitor of the mTOR (mechanistic target of Rapamycin kinase) signaling pathway (**Benyoucef A et al. 2015**). In addition, FOXP1 (forkhead box P1) is activated by PUM1 / 2 (pumilio RNA binding family member 1/2) and is also involved in the proliferation of HSC by decreasing the expression of p21 and p27 (**Naudin C et al. 2017).**

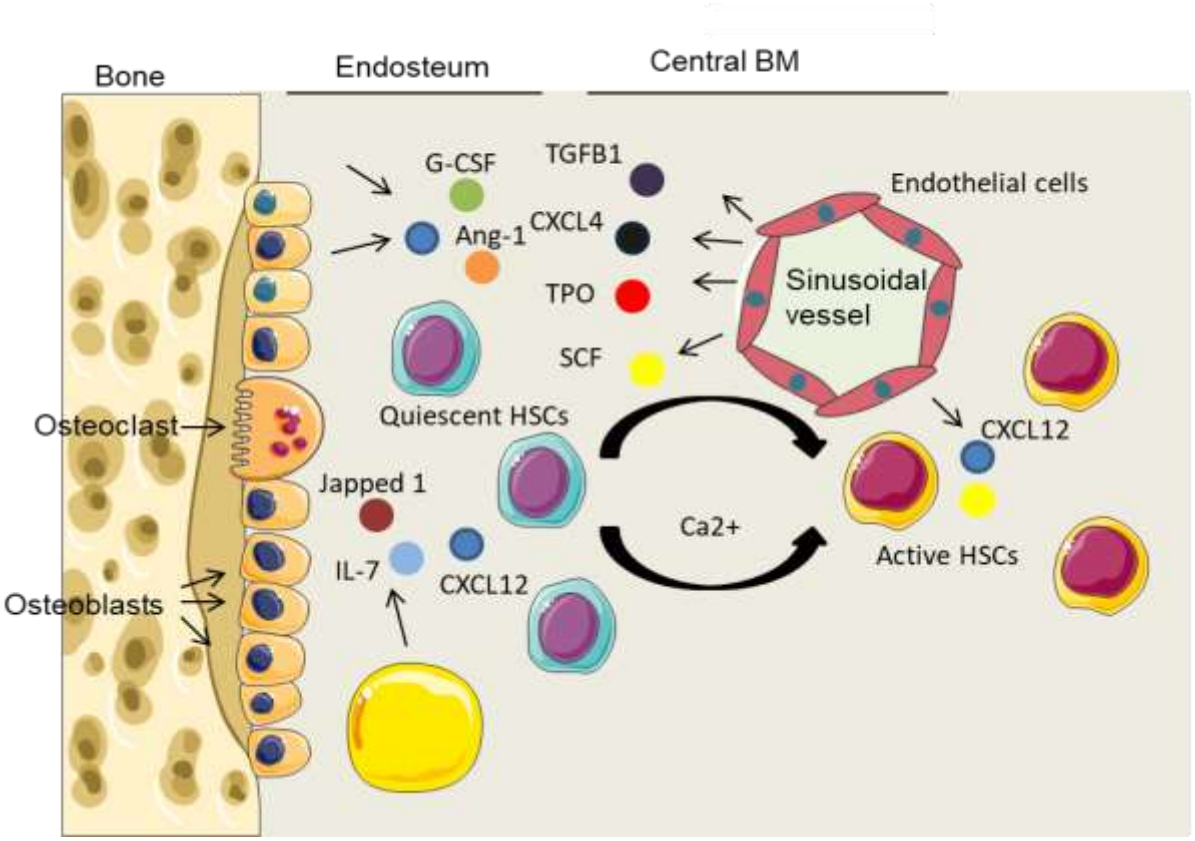

Figure 3: The two medullary niches have complementary functions. The endosteal niche (left) keeps HSC in quiescence thanks to the production of several factors and a hypoxic environment. The sinusoidal niche (right) induces the proliferation of HSCs. The balance between quiescence and proliferation is maintained thanks to continuous exchanges of factors between the two niches ,especially when niches evolve in response to stress.IL7:Interleukin 7,SCF:Stem cell factor.Ang-1:Angiopoetin 1,TPO:Thrombopoeitin, G-CSF: Granulocyte colony stimulating ,HSC:hematopoietic stem cell. Adapted from (Wei Q and Frenette P. 2018).

Globally, in bone marrow, most HSCs are quiescent and only 20% of them are active and are in the G1 phase of the cell cycle (**Kunisaki Y et al. 2013**). In order to maintain their numbers constant throughout life, HSCs have the potential to divide asymmetrically. One of the two daughter cells then remains in contact with the niche and retains its primitive character while the second embarked on a path of differentiation (**Inaba M and Yamashita Y. 2012**; **Ting SB et al. 2012**). Hematopoietic differentiation is therefore a finely regulated process.

1.3 Development of T cells (T lymphocytes)

T cells are derived from hematopoietic stem cells that are found in the bone marrow. Lymphoid progenitors which have developed from hematopoietic stem cells in the bone marrow migrate to the thymus and undergo a series of maturation steps that can be identified based on the

expression of different cell surface markers, to become functional T cells **(Brown G et al. 2018** and **Krueger A et al. 2017**). In the thymus, thymocytes express their specific T cell markers, including TCR, CD3, CD4 or CD8, and CD2. T cells also undergo thymic education through positive and negative selection.

The thymus is made up of an outer cortex and an inner medulla region. Developing thymocytes interact with the thymus stromal (cortical epithelial) cells, and undergo proliferation. The developmental stages of thymocytes started in the cortex and continue in medullary thymic areas after migration of thymocytes into the latter. The earliest developing thymocytes begin to express CD2 but lack the expression of CD4 and CD8 (the markers for Th and Tc lineages) and are termed double negative (DN) cells. The figure below (**Figure 4)** shows the ordered expression of these markers. The majority of DN cells in the thymus give rise to mature αβ T cells, and approximately 5% bear the γδ T cell receptor (TCR).

DN cells undergo a TCR β gene rearrangement (**Franchini DM et al. 2009**). The coupling of TCR β chain with pre-Tα and CD3 molecules to form the pre-TCR complex, represents a critical checkpoint in T cell differentiation known as β-selection. Indeed, cells that fails to generate a functionally rearranged TCR β chain at this stage of development and do not undergo successfully β selection will die by apoptosis. Signals derived from the pre-TCR complex, rescue thymocytes from apoptosis. It also stimulates them to proliferate, differentiate and express CD4 and CD8 to become double positive cells **(Rothenberg E et al. 2008)**.

DP cells rearrange their TCR-α chain loci, to produce an αβ-TCR .Double positive αβ cells move into the cortico-medullary junction, where they undergo a “thymocyte selection”, negative and positive selection. Selection depends on the affinity of the thymocyte expressed TCRs for self-peptides bound to major histocompatibility complex (pMHC) class I or class II molecules. Positive selection occurs in the cortex, when the TCR–self-pMHC interactions that are between a minimum and maximum affinity threshold result in the transduction of sufficient TCR signals that promote the survival of DP cells and their maturation. However, if the TCR–self-pMHC interactions are below the minimum affinity threshold and cannot generate the TCR signals sufficient for positive selection, than DP cell undergoes death by neglect (**Gaud G et al. 2018**). Thymocytes then migrate into the medulla to undergo negative selection. Negative selection occurs, when the TCR–self-pMHC interactions that are of maximum affinity threshold, transduce strong signals leading to cell death by apoptosis (**Klein L et al. 2014**).

Following selection, down-regulation of co-receptor produces either naïve CD4+ or CD8+ single positive cells that exit the thymus and circulate the periphery. Positive selection on MHC Class I will produce a CD8 Tc cell, while positive selection on MHC Class II will yield a CD4 Th cell.

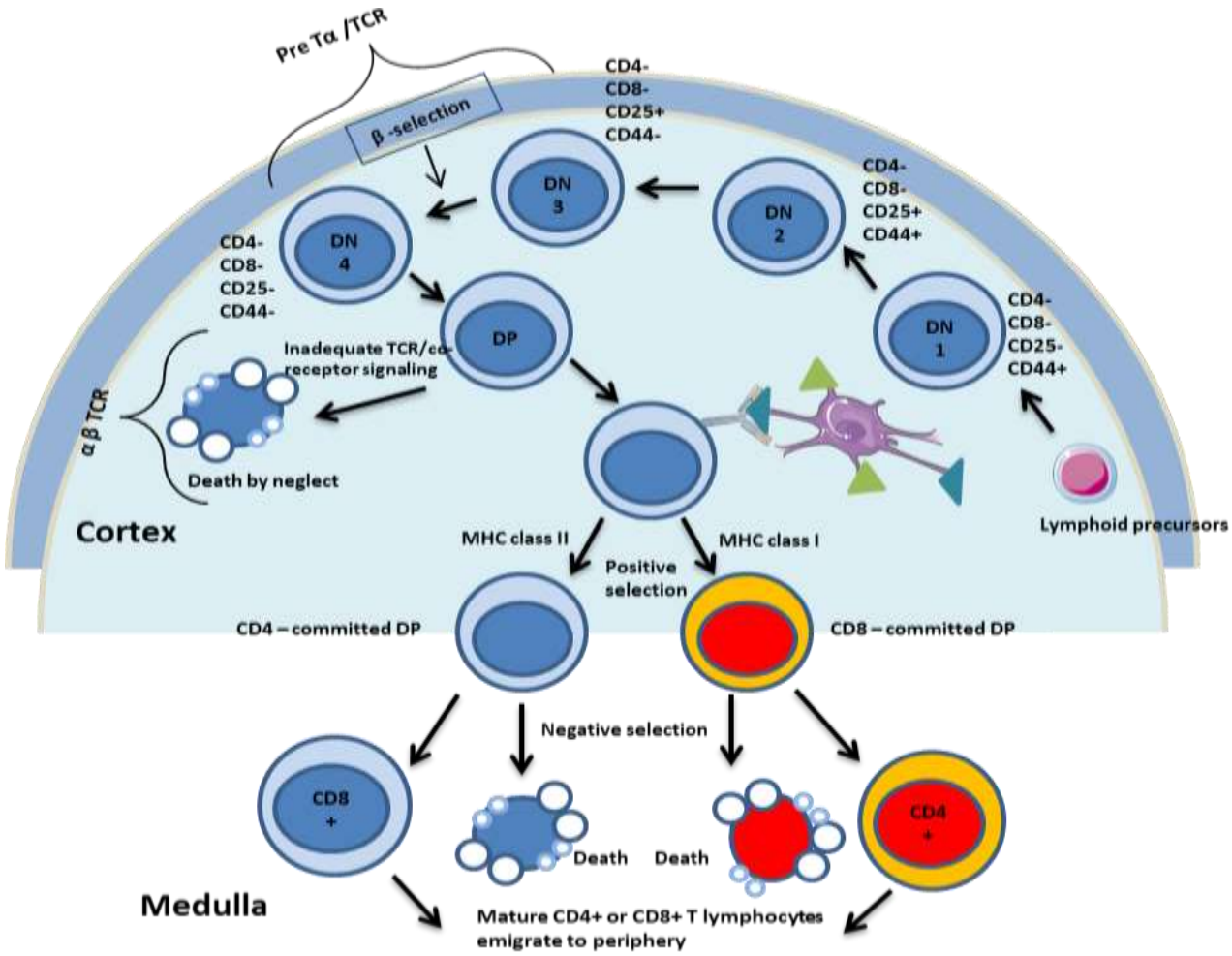

Figure 4: Overall scheme of T-cell development in the thymus. Committed lymphoid progenitors arise in the bone marrow and migrate to the thymus. The earliest developing thymocytes begin to express CD2 but lack the expression of CD4 and CD8, and are termed double negative (DN). DN thymocytes can be further subdivided into four stages of differentiation (DN1, CD44+CD25− ; DN2, CD44+CD25+; DN3, CD44− CD25+; and DN4, CD44− CD25− . As cells progress through the DN2 to DN4 stages, they express the preTCR, which is composed of the non-rearranging pre-Tα chain and a rearranged TCR β-chain. Successful pre-TCR expression leads to substantial cell proliferation during the DN4 to double positive (DP) transition and replacement of the pre-TCR α-chain with a newly rearranged TCR α-chain, which yields a complete αβ TCR.Double positive T cells interact with self-antigens in the context of major histocompatibility complex (MHC) class I or class II molecules. The fate of the DP thymocytes depends on signaling that is mediated by interaction of the TCR with these self-peptide–MHC ligands. Too much signaling can promote acute apoptosis (negative selection). The appropriate, intermediate level of TCR signaling initiates effective maturation (positive selection). Thymocytes that express TCRs that bind self-peptide–MHC-class-I complexes become (SP) CD8+ T cells, whereas those that express TCRs that bind self-peptide–MHC-class-II ligands become (SP) CD4+ T cells, which exit the thymus and circulate the periphery. SP: single positive, DP: double positive, DN: double negative. Drawn according to information from (Germain RN. 2002).

1.4 Development of B cells (B lymphocytes)

B cells develop from hematopoietic stem cells (HSCs) that originate from bone marrow. HSCs first differentiate into multipotent progenitor (MPP) cells, then common lymphoid progenitor (CLP) cells then B cells (**Kondo M. 2010**). B cell development begins in the fetal liver and continues in the bone marrow .The bone marrow is the primary location for early B cell development from stem cell to immature B cell while the development from immature to mature B cell takes place in secondary lymphoid organs such as the spleen and lymph nodes (**Lim VY et al. 2017**). Development progresses through the pro-B-cell, pre-B-cell and immature-B-cell stages. Each of them is characterized by different gene expression patterns and immunoglobulin H chain and L chain gene loci arrangements, the latter due to B cells undergoing V(D)J recombination during their development.

So during this differentiation, rearrangements at the immunoglobulin locus result in the generation and surface expression of the pre-B-cell receptor (pre-BCR), which is comprised of an Igμ heavy chain and surrogate light chains (VpreB or Vλ5) and finally a mature BCR (comprised of rearranged heavy- and light-chain genes) that is capable of binding antigen. At this immature stage of development, B cells undergo positive selection when the pre-B receptor binds its ligand and they undergo negative selection when they bind strongly to multivalent ligands. The BCRs of immature B cells are tested for self-reactivity by the surrounding tissue in the bone marrow. If the BCR can bind strongly to self-antigen, then the B cell undergoes one of these fates: clonal deletion, receptor editing or anergy **(Crosby L and Elliott A. 2019)**. Together these mechanisms create so called central tolerance in which the mature B cells don't bind with self-antigens present in the bone marrow. Also there are similar mechanisms in the periphery to induce tolerance to remaining self-reactive B cells and these mechanisms are responsible for peripheral tolerance (**Rice J et al. 2005**).

To complete development, immature B cells which successfully transit negative selection in the bone marrow are able to home via the blood to peripheral lymphoid organs such as the spleen. Immature B cells in the spleen are termed transitional B cells because they are transitioning from immature to mature B cells .They are subdivided into three distinct subsets based on termed transitional 1 (T1), transitional 2 (T2), and transitional 3 (T3). These cell types are differentiated based on differential surface expression of AA4, CD23, IgM and CD21/35 (**Allman D and Pillai S. 2008**).

T1 B cells transition to T2 B cells , T2 B cells which undergo proliferation and differentiation into follicular mature (FO) B cells, or into marginal zone (MZ) B cells depending on signals received through the BCR and other receptors (**Cerutti A et al.2013**). (T3) B cells are thought to represent primarily self-reactive anergic B cells (also known as An1 B cells) (**Figure5**) (**Cambier J et al.2007**) .

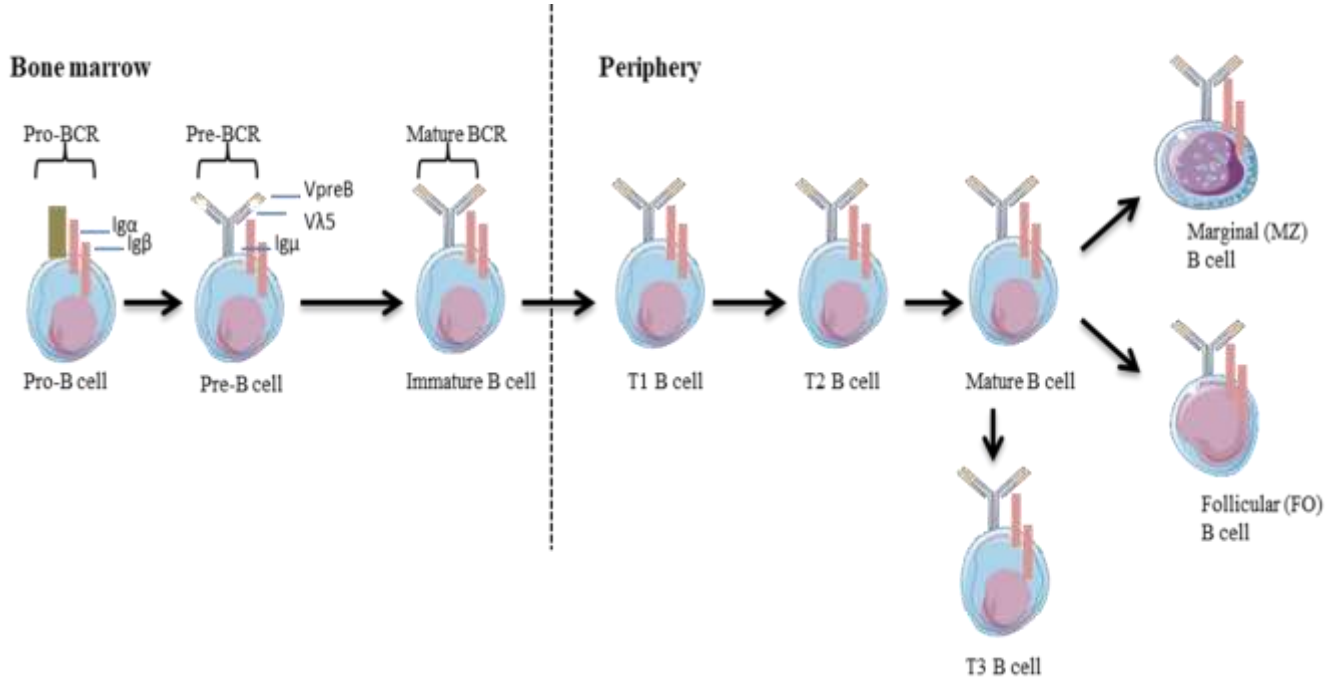

Figure 5: Overall scheme of B-cell development in the bone marrow and the periphery. The bone marrow is the primary location for early B cell development from stem cell to immature B cell while the development from immature to mature B cell takes place in secondary lymphoid organs such as the spleen and lymph nodes. Development progresses through the pro-B-cell, pre-B-cell and immature-B-cell stages. During this differentiation, rearrangements at the immunoglobulin locus result in the generation and surface expression of the pre-B-cell receptor (pre-BCR, which is comprised of an Igμ heavy chain and surrogate light chains (VpreB or Vλ5)) and finally a mature BCR (comprised of rearranged heavy- and light-chain genes) that is capable of binding antigen. At this immature stage of development, B cells undergo positive and negative selection. Cells successfully completing this checkpoint leave the bone marrow as transitional B cells, eventually maturing into mature follicular B cells (or marginal-zone B cells). Following an immune response, antigen-specific B cells develop into either plasma (antibody-secreting) cells or memory B cells. Transitional 3 (T3) B cells, once thought to be part of the linear development from immature to mature B cells, are now thought to represent primarily self-reactive anergic B cells (also known as An1 B cells). Adapted from (Cambier JC et al. 2007).

1.5 Regulation of hematopoietic differentiation

Numerous studies have been carried out to characterize the transcription factors and cytokines involved in hematopoietic differentiation. New techniques for studying individual cells make it possible to refine the analyzes by overcoming the heterogeneity of the population studied in order to better establish the role of these elements at the cellular level. This section presents a non-

exhaustive list of the most important transcription factors and growth factors implicated in hematopoiesis and leukemia.

1.5.1 Transcription factors

Because of their ability to regulate the expression of genes encoding cytokines or cytokine receptors, transcription factors control the proliferation and differentiation of progenitors into hematopoietic cells **(Figure 6)**.

The transcription factor TAL1 is one of the main regulators of hematopoiesis. It is required for erythroid progenitors differentiation and megakaryocytic progenitors differentiation. An abnormal high level of TAL1 expression was found in approximately 60% of T-ALL. The deletion or the lack of expression of TAL1, results in impaired homeostasis and even in early embryonic death. Moreover, the inhibition of TAL1 expression induces the apoptosis of blast cells of T-cell leukemia (**Vagapova E et al. 2018**).

RUNX1 (runt related transcription factor 1) controls the expression of many genes specific to hematopoietic lineages, involved in the differentiation and function of hematopoietic cells. High levels of RUNX1 are frequently observed in AML, T-ALL and B-ALL **(Michael Lie-a-ling et al.2020**). Deletion of RUNX1 in hematopoietic stem/progenitor cells causes defects in lymphoid and megakaryocytic (MK) development (**Chou BK et al. 2015** and **de Bruijn M and Dzierzak E. 2017**).

BCL11B plays roles in the brain and many other tissues, in addition to T cells, where it plays an important role in their development, proliferation, differentiation, and survival **(Huang X et al.2012**). High levels of BCL11B have been reported in the majority of T-ALL and T-cell lymphoma cell lines. Accordingly, deletion of BCL11B selectively induces apoptosis in malignant T cells while normal mature T cells remain unaffected (**Grabarczyk P et al. 2007**).

GATA-2 is expressed by HSC, MPP, MEP and ErP (erythroid progenitors). It is a key regulator of HSC engagement in the MEP lineage (**Nimmo R et al. 2015; Katsumura K and Bresnick E. 2017**). GATA2 was found highly expressed in 87% of AML patients (**Vicente C et al. 2012**). Consequently, GATA2 knockdown impedes cell proliferation and induces apoptosis of THP1 AML cells (**Menendez-Gonzalez et al. 2019**).

C / EBPα (CCAAT-enhancer binding protein) participates in maintenance of HSC but is mainly involved in the differentiation of myeloid cells. By inducing the expression of a specific genetic program for this lineage, it initiates the engagement of HSCs in myeloid differentiation (**Avellino R and Delwel R. 2017**). It is downregulation contributes to the development of AML (**Song G et al.2015)**.

PLZF is expressed at early stages of brain development and involved in the formation of deep layer cortical neurons. It is essential for the development of innate T cells (natural killer T cells) (**Puszyk W et al. 2013**). In T-ALL, PLZF was highly expressed in 50% of patients (**Jeon Y et al. 2012**), while, it was downregulated in 90% of B cell chronic lymphocytic leukemia (B-CLL) patients **(Parrado A et al. 2000**).

PU.1 (purine rich box 1), also called SPI-1 (spleen focus forming virus proviral integration site 1) belonging to the ETS family (E-twenty six) is a key regulator of hematopoietic differentiation. It has a more important role for the fate of HSC and progenitor populations than for the final stages of maturation of hematopoietic cells (**Burda P et al. 2010**). PU.1 acts in combination with other transcription factors such as GATA-1, RUNX-1, SP-1 (specificity protein 1), C / EBPα or IRF-4 (interferon regulatory factor 4), controlling the expression of many genes involved in myelopoiesis. The deletion or the reduction in PU.1 expression leads to the development of AML (**Takei H et al. 2019).**

NOTCH1 is a receptor which, following its cleavage and association with transcription factors, can regulate them. NOTCH1 consists of a long extracellular heterodimerization (HD) domain, a transmembrane domain and an intracytoplasmic domain. Its extracellular part is capable of binding to two families of transmembrane proteins: Jagged1 / 2 and Delta-like (DLLA, DLL3 and DLL4), expressed in particular by the cells of the bone marrow and the thymic cortex (**Radtke F et al. 2004; Koch, Fiorini et al. 2008**). Force generation is required for Notch receptor proteolysis with the γ-secretase enzyme ultimately freeing the NICD (NOTCH intra cellular domain) from the membrane (**Kovall et al. 2017**). NICD is translocated in the nucleus where it binds to RBPJκ and proteins of the MAML family (Mastermind-like family) in order to form a transcription activator complex capable of inducing the expression of several target genes specific for the T lymphocyte lineage **(Hosokawa and Rothenberg E et al. 2020).**

Thus, the NOTCH1 pathway is one of the important signaling pathways required for T cell development, and the thymus has crucial role of providing NOTCH ligands to progenitor T cells. Deletion of NOTCH1 in Hematopoietic stem/progenitor cells (HSPCs), or of the gene encoding the

Notch ligand Delta-like 4 (Dll4) in thymic epithelium, leads to a complete block of T cell development accompanied by the appearance of B cells in the thymus.

Ikaros (IKZF1) functions as tumor suppressors and to enforce developmental checkpoints specifically by making the activation and repression of different waves of regulatory genes more switch-like, rather than gradual, from one developmental stage to the next during the development of T and B lymphocytes (**Heizmann B et al. 2018)**. Deletions of IKZF1 (ΔIKZF1) resulted in human malignancies like pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL) and T-ALL (**Olsson L and Johansson B.2015)**.

Finally, PAX5 (paired box 5) is exclusively expressed by B lymphocytes. Acting in collaboration with EBF1 (early B cell factor 1) and TCF3 (transcription factor 3), it represses genes specific for other lineages such as NOTCH1 and induced the expression of a genetic program necessary for the development of B lymphocytes **(Medvedovic J et al. 2011**). PAX5 deletion leads to B-ALL development in humans, It activates the cell cycle of B cells and increases their ability to initiate leukemia **(Liu GJ et al. 2014)**.

1.5.2 Growth factors

Hematopoiesis is also regulated by growth factors belonging to the cytokine family. They are produced by spinal cord cells, lymphocytes and monocytes, with the exception of erythropoietin (EPO) secreted mainly by renal peritubular cells.

Cytokines generally activate survival and proliferation signals. Some act in a broad way, on the cells of several lineages at different stages of maturation, this is the case of the SCF (stem cell factor) which regulates the HSCs and the progenitors. Others like IL-7 or GM-CSF (granulocyte / macrophage colony stimulating factor), IL-6 and IL-3 have a more limited effect. G-CSF (granulocyte-CSF) has an action only on granulocyte differentiation. M-CSF (macrophage-CSF) and CSF-1 (colony stimulating factor 1) act on the differentiation of monocytes, EPO on the erythroid lineage and the thrombopoietin (TPO) on HSCs and on the megakaryocyte lineage. By attaching to their specific receptors, growth factors induce signals essential for hematopoiesis.

Deregulation of one of these signaling pathways can lead to malignant hemopathies such as Myeloproliferative Neoplasms (MPNs).

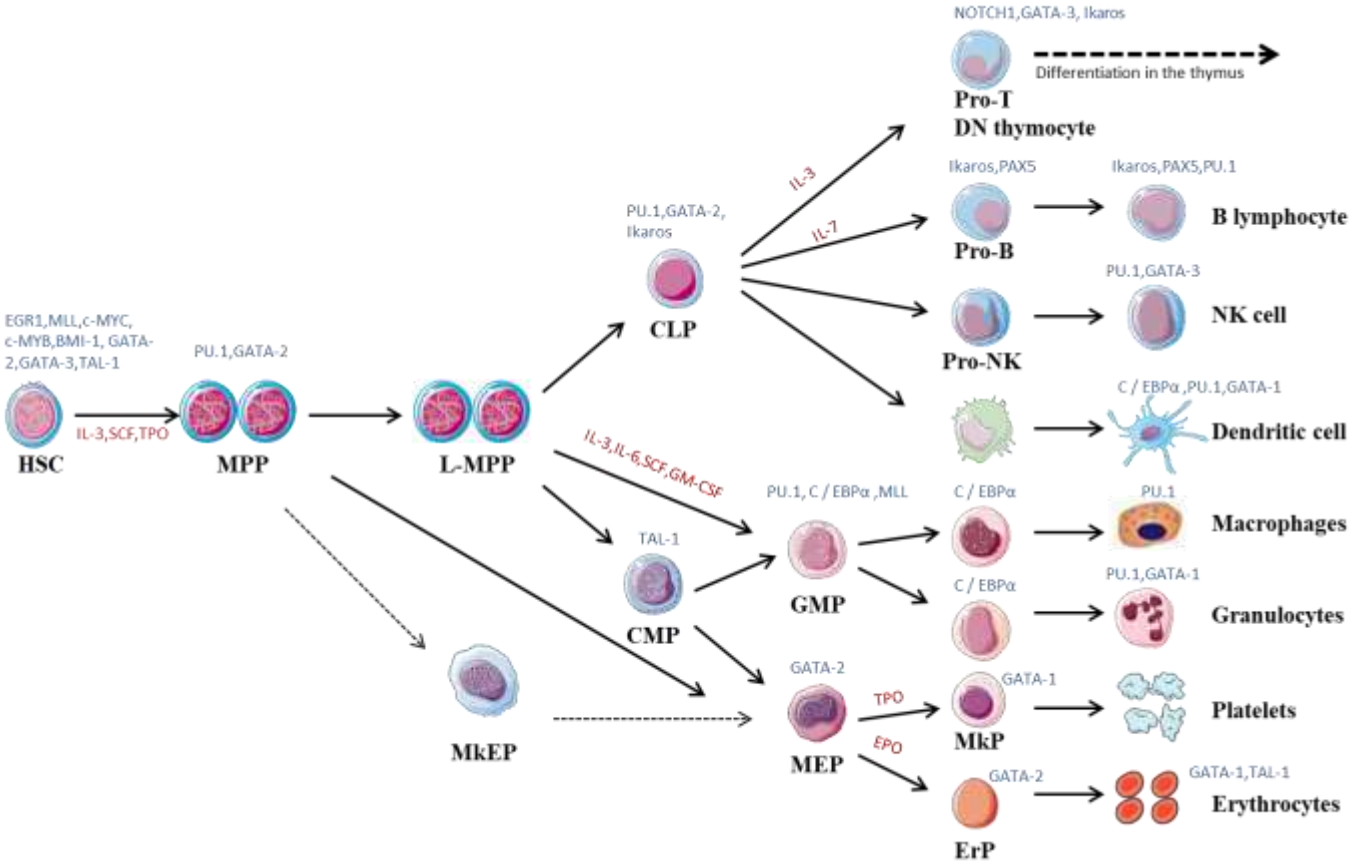

Figure 6: Transcription and growth factors involved in hematopoiesis. The fate of HSCs is regulated by the expression of transcription factors (in blue) and growth factors (in red) that control their differentiation. CLP: common lymphoid progenitors, CMP: common myeloid progenitors, DN: double negative, ErP: erythroid progenitors GMP: granulocyte / monocyte progenitors, HSC: hematopoietic stem cell, LMPP: lymphoid primed multipotent progenitors, MEP: megakaryocyte / erythroid progenitors, MkP: megakaryocyte-committed progenitors, MPP: multipotent progenitors. Drawn with information from (Grabher C et al. 2006, Ho I et al. 2009, Nimmo R et al. 2015, Burda, Avellino R and Delwel R. 2017, de Bruijn M and Dzierzak E.2017, Heizmann B et al. 2018)

2 Leukemia

As we mentioned in the “hematopoietic lineages” section, blood is a specialized body fluid which contains several broad categories of cells: Red Blood Cells (RBCs), White Blood Cells (WBCs), and platelets. Generally, leukemia refers to cancers of the WBCs. Leukemic WBCs suffer from a proliferation and differentiation disorder so they divide too quickly. However, these abnormal cells can’t fight infection as normal white blood cells do. Also they are resistant to apoptosis and they go into an unlimited process of self-renewal.

2.1 Types of leukemia

Leukemia is classified by the type of white blood cells affected (**Ciesla B. 2007**) and by how quickly the disease progresses (**Bain BJ. 2010**). The first group, classified according by how fast it develops, is divided into acute and chronic leukemia. Acute leukemia (fast-growing) is rapidly progressing and results in the accumulation of immature cells (blasts) in the bone marrow, which prevent the bone marrow from producing healthy hematopoietic cells. Chronic leukemia (slow-growing) progresses more slowly and results in the accumulation of cells with various levels of differentiation beyond the blast stage, even if these cells are relatively mature white blood cells but are abnormal.

The second group, classified according to which type of white blood cells is affected, is divided into lymphocytic and myelogenous leukemia. Lymphocytic (or lymphoblastic) is a malignant transformation of lymphoid progenitor cells and can be classified into B-cell and T-cell neoplasms. Myelogenous (or myeloid) leukemia is a very heterogeneous disease caused by malignant transformation of myeloid progenitor cells that create red blood cells, platelets and other kinds of white blood cells (granulocytes and macrophages).

The four main types of leukemia are:

Acute lymphocytic leukemia (ALL), also called acute lymphoblastic leukemia, is a blood cancer that results when abnormal white blood cells (leukemic cells) accumulate in the bone marrow. ALL progresses rapidly, replacing normal blasts that produce functional lymphocytes with leukemic cells that are immature. Blood carries the leukemic cells to the brain, liver and spleen where they continue to divide and spread (**www.LLS.org**).

ALL affects B cells more than T cells. ALL is the most common type of leukemia in young children (under 15) but it can also occurs in adults (more than 45).

Acute myeloid leukemia (AML), also known as acute myeloblastic leukemia, acute granulocytic leukemia or acute no lymphocytic leukemia is a fast-growing form of cancer of the blood and bone marrow.

AML is the second most common form of childhood leukemia and one of the most common forms for adults. It progresses rapidly, replacing normal blasts that produce functional red, white blood cells and platelets with immature leukemic cells that are unable to fend off infection.

AML has eight different subtypes, which are based on the cell that the leukemia developed from. The types of acute myelogenous leukemia include (**Setiawan A et al. 2018**):

- Myeloblastic (M0)
- Myeloblastic (M1)
- Myeloblastic (M2)
- Promyelotic (M3)
- Myelomonocytic (M4)
- Monocytic (M5)
- Erythroleukemia (M6)
- Megakaryocytic (M7)

Chronic lymphocytic leukemia (CLL) is a very common form of adult leukemia; it progresses slowly, replacing normal blasts that produce functional lymphocytes with leukemic cells that are immature. In CLL, the abnormal lymphocytes take longer to develop and multiply, which is why some cases stay stables for years and don't need treatment.

Chronic myeloid leukemia (CML) also known as chronic myelogenous leukemia, it is the most common form of leukemia for elderly people. CML is a form of cancer that affects the bone marrow and blood. It begins in the myeloid cells of the bone marrow and then, over time, spreads to the blood and to other areas of the body. CML has been shown to be associated with an abnormal chromosome known as the Philadelphia chromosome (Ph chromosome) (**Kang ZJ et al.2016**).

In my thesis, I worked on T-cell and B-cell acute lymphoblastic leukemia, so I chose to highlight only Acute Lymphoblastic Leukemia.

2.2 Clinical presentation of ALL

Patients with acute lymphocytic leukemia (ALL) appear with signs and symptoms related to direct infiltration of bone marrow and other organs by leukemic cells, or in connection with a decrease in the production of normal hematopoietic elements. The duration of symptoms in patients with ALL can range from a few days to several months.

Figure 7 shows the common symptoms of acute lymphoblastic leukemia that could affect the, muscles, skin, bones, spleen, liver, testicles and CNS (**Paul S et al. 2016**).

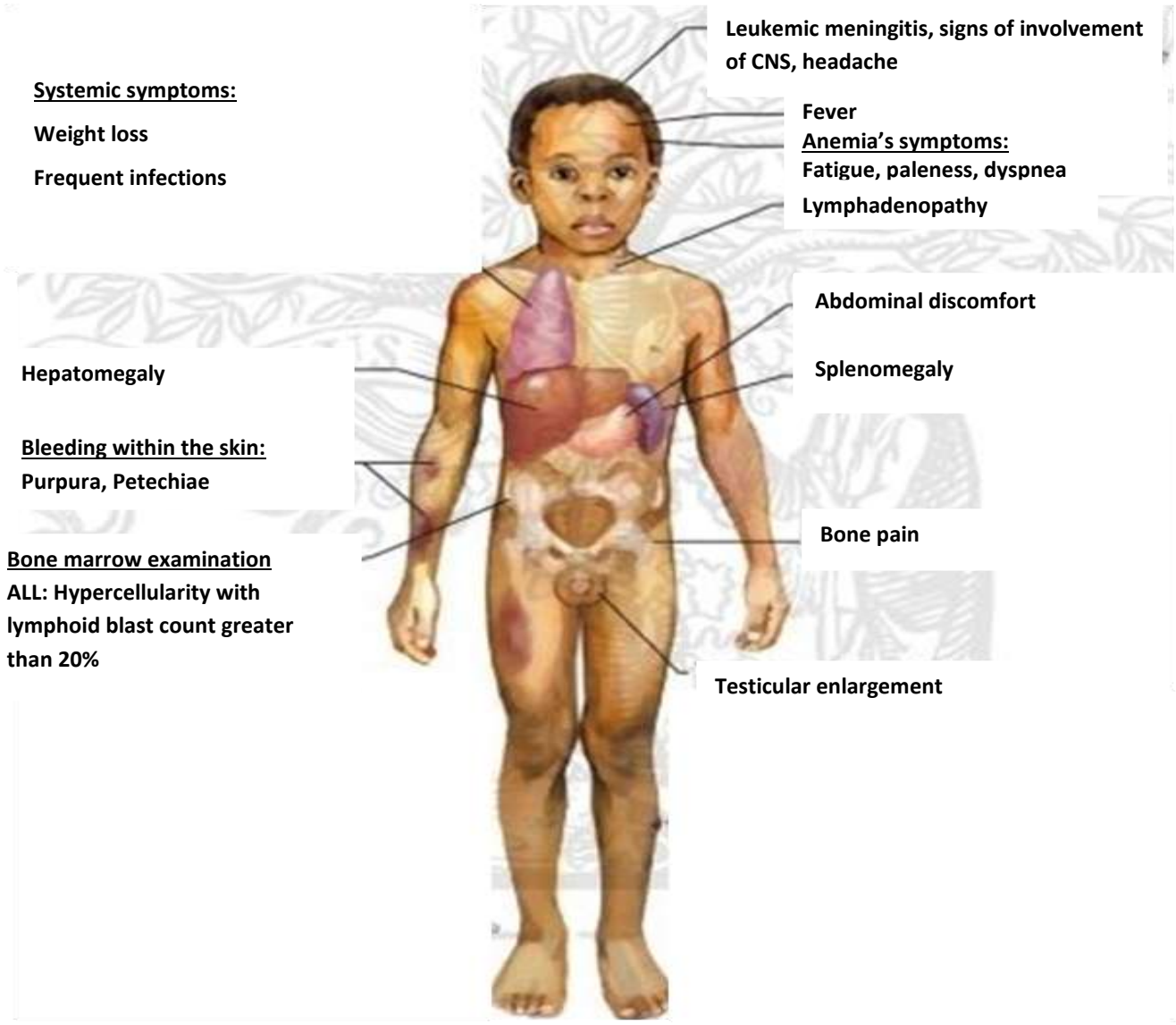

Figure 7: Clinical presentation of acute lymphoblastic leukemia.

2.3 Risk Factors for Acute Lymphocytic Leukemia (ALL)

Environmental, biological and genetic factors play a role in leukemia susceptibility and development. Exposure to infections has been widely studied as causal mechanisms for the leukemia development: lack of infections during early life cause dysregulation in immune system, which leads to an abnormal response to common infections later in childhood (**Greaves M. 2018**).

In addition, a virus called HTLV-1 (human T cell leukemia virus) increases the risk of developing the adult T-cell leukemia (ATL). Also, Epstein-Barr virus has been definitively linked to the development of ALL.

Several studies have examined possible genetic, infectious, and environmental risk factors to determine the etiology of acute lymphoblastic leukemia. In general, benzene and ionizing radiation are two environmental exposures strongly associated with the development of AML or ALL (**Belson M et al. 2007; Cangerana Pereira F et al. 2017** and **Zhao J et al. 2020)**. Even though leukemia is not considered as an inheritable disease, there are some genetic factors that contribute to disease onset, like Down's syndrome, Fanconi anemia, ataxia telangiectasia and Bloom syndrome (**McGee RB et al. 2016)**. There are also a number of genes whose mutation predisposes carriers to ALL development (e.g. *ARID5B, CEBPE, GATA3, ETV6, IKZF1* and *PAX5*) (**Inaba H et al. 2020**).

2.4 Diagnosis of Acute Lymphoblastic Leukemia

An accurate diagnosis of acute leukemia is crucial to help the doctor assess the progression of the disease and determine the appropriate treatment. First, the patient must undergo a routine physical examination and if signs and symptoms suggest that the person may have leukemia, doctors perform several laboratory tests. Tests and procedures used to diagnose acute lymphocytic leukemia include:

2.4.1 Complete Blood Count with Differential (CBC w/ diff)

This test measures the number of red blood cells, white blood cells and platelets in a sample of blood. It also measures the percentage of the different types of white blood cells (differential WBC count) in the sample and show the presence of immature blast cells normally found in the bone marrow. People with ALL may have several signs of bone marrow failure like anemia, abnormal leukocyte and differential counts, and thrombocytopenia (**Table1**).

Parameters	Reference interval in Healthy people	Results in ALL patients
Hemoglobin (HGB)	12.9 - 18.4 g/dL	<12 g/dL (Anemia)
Leukocytes (WBCs)	4.5 - 11.0 $x10^9$/L	> 11 $x10^9$/L (Hyperleukocytosis)
Neutrophils	2.0 - 6.0 $x10^9$/L	<1.2 $x10^9$/L (Neutropenia)
Platelets	150 - 450 $x10^9$/L	<150 $x10^9$/L (Thrombocytopenia)

Table1: Complete blood count in healthy people and in patients with ALL.

2.4.1 Bone Marrow Aspiration and Biopsy

During bone marrow aspiration, a specialized needle is used to remove a sample of bone marrow from the hipbone or breastbone (**Pui C et al. 2015**). Bone marrow aspirates are used to evaluate cell morphology and perform a cell count of different marrow elements, including blasts.

Bone marrow biopsy removes a small amount of bone filled with marrow. The biopsy allows the pathologist to visualize the microstructure of the marrow and describe overall cellularity, stromal elements, and the proportion and maturation of hematopoietic cells (**Ridgeway J A et al. 2017**).

2.4.2 Immunophenotyping

Beside cell assessment, immunophenotyping is performed using flow cytometry. It is a complement essential in order to confirm the cell lineages involved in ALL. The antigens, or markers/proteins, on the surface of the cells are sought (**Table 2**) (**Herold NC and Mitra P. 2020**). This examination helps to determine at what stage the blasts are blocked and assess their level of differentiation as well as determine if there is a B-cell lineage or T-cell lineage ALL. The results of the immunophenotyping will condition the treatment therapy, disease monitoring and evaluation of residual disease.

	High specificity	Medium specificity	Low specificity
B cell lineage	**CD79a cyto IgM cyto CD22**	**CD19 CD20 CD10**	**TdT CD24**
T cell lineage	**CD3 T cell receptor αβ T cell receptor γδ**	**CD2 CD5 CD7 CD8 CD10**	**TdT CD1a**
Myeloid lineage	**MPO**	**CD13 CD33 CD65w CD117**	**CD14 CD15 CD64**
Megakaryocytic markers	-	**CD41 CD61**	-
Monocytic markers	-	**CD14 CD11b CD64 CD36**	-
Hematopoietic precursor markers	-	**CD34 HLA-DR TdT**	**CD45**

Table 2: Antigens commonly used for flow cytometric lineage assignment

2.4.3 Genetic Tests

Genetics tests are other types of laboratory tests used for leukemia diagnosis (**Wan T et al. 2014**). Among these tests we can cite:

- Cytogenetic Analysis (Karyotyping) used to search for abnormal changes in leukemia cell chromosomes in ALL patients, and it also provides important information to determine the best treatment options for a patient.
- Fluorescence in situ Hybridization (FISH) used to detect certain abnormal changes in the chromosomes and genes of leukemia cells.

- The Polymerase Chain Reaction (PCR**)** is mainly used. Its sensitivity is very high and this makes it possible to assess the prognosis of each patient. In fact, more than 50% of ALL exhibit translocations inducing gene fusions that induce the appearance of abnormal proteins (**Figure 8**) (**Duployeza N and Preudhomme C. 2015**). Their identification makes it possible to determine the most suitable therapeutic management.

2.4.4 Imaging tests

Imaging tests such as MRI, computerized tomography (CT) scan, Ultrasonography and echocardiogram may help determine whether cancer has spread to the brain, spinal cord, liver, spleen or other parts of the body.

2.4.5 Spinal fluid test

A lumbar puncture test is used to collect cerebrospinal spinal fluid (CSF), which is a fluid that flows around the brain and spinal cord. The cerebrospinal fluid sample is tested to determine whether or not acute lymphocytic leukemia has spread to this region (**Del Principe MI et al.2014**).

2.5 Classification of ALL

In addition to morphology, immunophenotypic, cytogenetic and molecular analyses are needed to classify leukemia into different subtypes. Moreover, there are two classification systems which use laboratory hematology to categorize acute leukemia: The French-American-British (FAB) Classification System and World Health Organization classification system (WHO).

2.5.1 Immunophenotypic Subtype Classification of ALL

The immunophenotyping is very important for the diagnosis and classification of T-ALL and B-ALL. It helps to determine the cell lineage, degree of maturity and characteristics of the abnormal phenotype.

2.5.1.1 B-ALL classification

In the case of B-ALL, antibodies against human leukocyte antigen-DR (HLA-DR), CD19, CD10, CD20, Cμ and surface immunoglobulin M (IgM) are used to subdivide B-ALL into three subgroups: B-precursor ALL, pre- B ALL and B-ALL **(Table 3)**.

CD19, Cμ and surface Ig are used to distinguish between subgroups of B-ALL. B-ALL express CD19 and surface Ig, pre-B ALL express expresses CD19 and Cμ, while B-precursor ALL express only CD19. The nuclear enzyme Terminal deoxynucleotidyl transferase (TdT), CD10 and CD34 are used to determine the nature of ALL cells. TdT is seen in all B-Precursor ALL cases, and in some Pre-B ALL but not in B-ALL, CD10 is seen in all B-ALL cases, while CD34 is found in all B-precursor ALL and in B-ALL but not in Pre B-ALL. Many other antibodies and markers are used to classify B-ALL, including HLA-DR, CD20, CD24, CD22 and CD79/b **(Ramyar A et al. 2008)**

	Surface Ig	Cyto-μ	CD19	CD20	CD22	CD24	CD34	CD79a/b	HLA-DR	CD10	TdT
B-Precursor ALL	No	No	Yes	Yes/No	No	Yes	Yes	Yes	Yes	Yes	Yes
Pre-B ALL	No	Yes	Yes	Yes/No	No	Yes	No	Yes	Yes	Yes	Yes/No
B-ALL	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes/No	No

Table 3: Immunophenotypic classification of T-ALL.

2.5.1.2 T-ALL classification

In the case of T-ALL, antibodies against CD1, CD2, cytoplasmic CD3 (cCD3), surface CD3 (sCD3), CD4, CD5, CD7, CD8 and TdT are used to subdivide T-ALL into four immunophenotypes: pre-T cells, early cortical, late cortical and the medullary (mature) T cell phenotype (**Borowitz M et al. 2008**) **(Table 4)**.

The pre T- cells express CD7, cCD3 and TdT only. The early cortical T cells express CD2, CD5, CD7 and TdT; however, the late cortical T cells show CD1, CD2, CD5, CD7 and dual CD4/CD8 with low expression of sCD3. The mature T cells reveal CD2, CD5, CD7 and CD4 or CD8, without expressing TdT **(Follini E et al. 2020**). In addition, human leukocyte antigen-DR (HLA-DR) and CD10 were expressed more in adult T-ALL phenotypes than in children T-ALL phenotypes.

	CD1	CD2	cCD3	sCD3	CD4	CD5	CD7	CD8	TdT
Pre-T	No	No	Yes	No	No	No	Yes	No	Yes
Early cortical	No	Yes	Yes	No	No	Yes	Yes	No	Yes
Late cortical	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Medullary	No	Yes	Yes	Yes	Yes/No	Yes	Yes	Yes/No	Yes/No

Table 4: Immunophenotypic classification of T-ALL.

Recently, a new subgroup, ETP-ALL (Early-T Precursor), was represented, which shows distinct immunophenotypic features, namely impaired expression of CD5, expression of myeloid and/or a stem cell marker such as CD34, CD13 and CD33 without expression of CD1 and CD8 (**Chiaretti S et al.2014)**.

2.5.2 Cytogenetic abnormalities in ALL

There is less information on the cytogenetic abnormalities in marrow cells of patients with ALL than on abnormalities in other leukemias; nonetheless, some patterns of karyotypic change in ALL are evident. More than 50% of patients appear to have abnormal karyotype (**Perez-Vera P et al. 2004**). ALL can be classified by numerical or structural cytogenetic features **(Figure 8).**

2.5.2.1 B-ALL abnormalities

t (9;22)(q34;q11.2)/BCR-ABL1

The Philadelphia chromosome was first described by Nowell and Hungerford in 1960. It is found in Ph (+) B-cell acute lymphoblastic leukemia. t (9;22)(q34;q11.2)/*BCR-ABL1*, is the most frequent abnormality among adults with B-ALL which is detected in around 29% of patients (**Kang ZJ et al. 2016** and **Mancini M et al. 2005**). In contrast, the incidence of t (9; 22) in childhood is low (3-5%) (**Schultz KR et al. 2009**). Patients (both adults and children) with B-ALL and BCR-ABL translocation treated with chemotherapy and Dasatinib or Imatinib showed a better prognosis(**Takeuchi A et al. 2021**).

High hyperdiploidy

High hyperdiploidy is characterized by a non-random pattern of chromosomal gains (51 to 67 chromosomes), comprises the largest cytogenetic subgroup of B-cell acute lymphoblastic leukemia and is one of the most common malignancies in children (**de Smith A et al. 2016)**. The chromosomes most frequently gained are X, 4, 6, 10, 14, 17, 18 and 21 (**Chilton L et al. 2014**). The incidence of high hyperdiploidy in childhood B-ALL ranges from 16% to 27%. In adults the incidence of high hyperdiploidy is remarkably lower and accounts for between 5% and 6% of the diagnostic cases (**Woo JS et al. 2014**). This group is associated with good risk factors: age 2-9 years, leukocyte counts less than $10x10^9$/L, female sex, C-ALL immunophenotype, FAB type L1. Patients with high hyperdiploidy enjoy the best prognosis. An average event-free survival of 5 years is achieved in 72% of children. An initial report showed that the presence of structural chromosomal abnormalities (translocations, deletions and duplications) in addition to high hyperdiploidy have an adverse impact on prognosis, but this was not confirmed in a more recent report from the same group (**Pui CH et al.1989**).

t (12; 21) (p13; q22)/ETV6-RUNX1

t (12; 21) (p13; q22) translocation leading to ETV6-RUNX1 fusion is found in B-cell precursor acute lymphoblastic leukemia (BCP-ALL). It occurs in 25% of pediatric cases, but its incidence is very low in adults **(Al-Shehhi H et al. 2012)**. The prognosis of children with t (12; 21) is excellent (**Forestier E et al. 2008)**

t (4; 11)(q21; q23)/MLL-AF4

t (4; 11)(q21; q23)/MLL-AF4 is found in newly diagnosed B-cell acute lymphoblastic leukemia. The incidence of this translocation has been reported as 9 and 13% in children and adults (**Duployeza N and Preudhomme C. 2015**; **Moorman AV et al. 2007**). The t (4; 11) predicts a poor prognosis in both children and adults.

Hypodiploidy

Hypodiploidy is defined as a clonal loss of at least one chromosome. In B-ALL it occurs in between 5% and 8% of childhood patients. It may be subdivided into high hypodiploidy (40–45 chromosomes) and low hypodiploidy (33–39 chromosomes) with distinct genetic and clinical features. In some surveys hypodiploidy cases have shown a better prognosis than average others they have had a worse prognosis (**Harrison CJ et al. 2004**).

Near Triploidy/Tetraploidy

Near-triploidy is defined as the presence of more than 65 chromosomes (66-80 chromosomes) and near-tetraploidy (81-102 chromosomes). It is strongly associated with the ETV6-RUNX1 fusion in B-lineage leukemic cells (**Coccé M et al. 2015**). The frequency of Near Triploidy/Tetraploidy was respectively 7% in adults and only 2% of children with ALL. The prognosis in adults and in children is good (**Charrin C et al. 2004**).

t(1; 19) (q23; p13) and der(19)t(1; 19)(q23; p13)/E2A-PBX1

Translocation (1; 19) (q23; p13.3)/*TCF3 (E2A)-PBX1* is one of the most frequent translocations in B-acute lymphoblastic leukemia (B-ALL). This translocation can occur in a balanced t (1; 19)(q23;p13) or unbalanced der(19)t(1;19)(q23; p13) form and can result in the fusion of *TCF3* (transcription factor 3) found at 19p13 and *PBX1* (pre-B cell leukemia homeobox 1) found at 1q23 to form a chimeric gene whose protein product alters cell differentiation arrest, among other cellular processes **(Tirado C et al. 2015)**. This translocation is found in between 2% and 9% of ALL (**Burmeister T et al. 2010**). It occurs in 3% of adult (**Moorman AV et al.2007**) and in 6% of pediatric ALL. The prognostic significance of this abnormality was unclear. However, a recent study has suggested that an intensive treatment with CVAD regimen could markedly improve prognosis (**Garg R et al. 2009**).

Near haploidy

Near haploidy is defined as a chromosomal gain from the haploid number of 23 chromosomes. The chromosomes frequently gained are 10,14,18,21. Both sex chromosomes are frequently present. The incidence of near haploidy in B-ALL is between 0.5% and 2.5%. The prognosis for cases with near haploidy is poor (**Raimondi SC et al. 2003**).

2.5.2.2 T-ALL abnormalities

Pseudodiploidy

Pseudodiploidy is defined as the presence of 46 chromosomes and a structural change or loss of 1 or more chromosomes complemented by gain of the same number of other chromosomes. Pseudodiploidy can be identified only through rigorous karyotype analysis. In T- ALL, the majority of patients have pseudodiploid karyotypes (**Mrózek K et al. 2008**).

t (1; 7) (p32; q35) , t (7; 9) (q34; q34) , t (7;11) (q35; p13) and t (7; 19)(q35 ;p13)

These translocations are found in T- ALL, the translocations share a common breakpoint at 7q 32-36, at the site of the T-cell receptor genes. In the t (1; 7) (p32; q35) the gene involved is *TAL-1*, in the t (7; 9) (q34; q34) the gene involved is *NOTCH1*, in the t (7; 11) (q35; p13) the *TTG2* gene is involved and in the t (7; 19) (q35; p13) the *LYL1* gene is involved. The incidence of t (1; 7), t (7; 11) and t (7; 19) has been reported as less than 1% to each incidence in adults and children. However, the incidence of t (7; 9) NOTCH1 translocation has been reported as 1% of T-ALL patients, while NOTCH1 activation is detected in about 60% of T-ALL (**Tosello V et al. 2013)**

t(1; 14) (p33; q11) , t (8; 14) (q24; q11) , t (10;14) (q24; q11.2) and t(11 ;19) (q23 ;p13)

These translocations are also found in T- ALL, they share a common breakpoint at 14q11, at the site of the T-cell receptor genes TCRA. In the t (1; 14) (p33; q11) the gene involved is *TAL-1*, in the t (8; 14) (q24; q11) the gene involved is the oncogene *c-MYC*, while in t (10; 14) (q24; q11.2) the *HOX11* (*TLX1)* gene is involved and in t (11; 19) (q23; p13) the MLL gene is involved (**Ballerini P et al. 2002**). The incidence of t (1; 14) has been reported as 3 and 2.5 % in children and adults, the incidence of t (8; 14) has been reported as 2 and 4 % in children and adults. In contrast, the incidence of t (10; 14) in childhood is very low (0.5%), while in adults the incidence of this translocation is around 8%. The incidence of t (11; 19) is very low in children (0.3%) and adults (0.5%). Prognosis for the patients with t (1; 14) and t (8, 14) translocations is poor while t (10; 14) is associated with a favorable outcome.

2.5.2.3 Abnormalities common in T-ALL and B-ALL

Del 6q, Del 9p and Del 12p

Del 6q: Deletion of 6q is found in T and early B-lineage ALL. It involves breakpoints between 6q13-6q21 and 6q21-6q23.

Del 9p: the deletion of the short arm of chromosome 9 (9p) is found in T and early B-lineage ALL. The deletion is associated with the loss of the interferon genes INFA, INFB and the multiple tumor suppresser genes MTSI and MTS2. This abnormality has been associated with “lymphomatous disease”: lymphadenopathy, splenomegaly, CNS involvement and high leukocyte count (**Nahi H et al. 2008**). Also, 9p deletions can result in the loss of CDKN2A and CDKN2B genes (**Girardi T et al. 2017)**. It is present in up to 70% of pediatric T-ALL cases.

Del 12p: The abnormalities of 12p include deletions, balanced and unbalanced translocations. It is now becoming clear that a proportion of the 12p deletions may now be reclassified as translocations. t (12; 21) translocation results in the fusion of the AML1 gene located at 21q22 and the TEL gene located at 12p13. It is associated with a very good prognosis. The projected event-free survival has been shown to be 74% at 3 years (**Wiemels JL et al. 2008**).

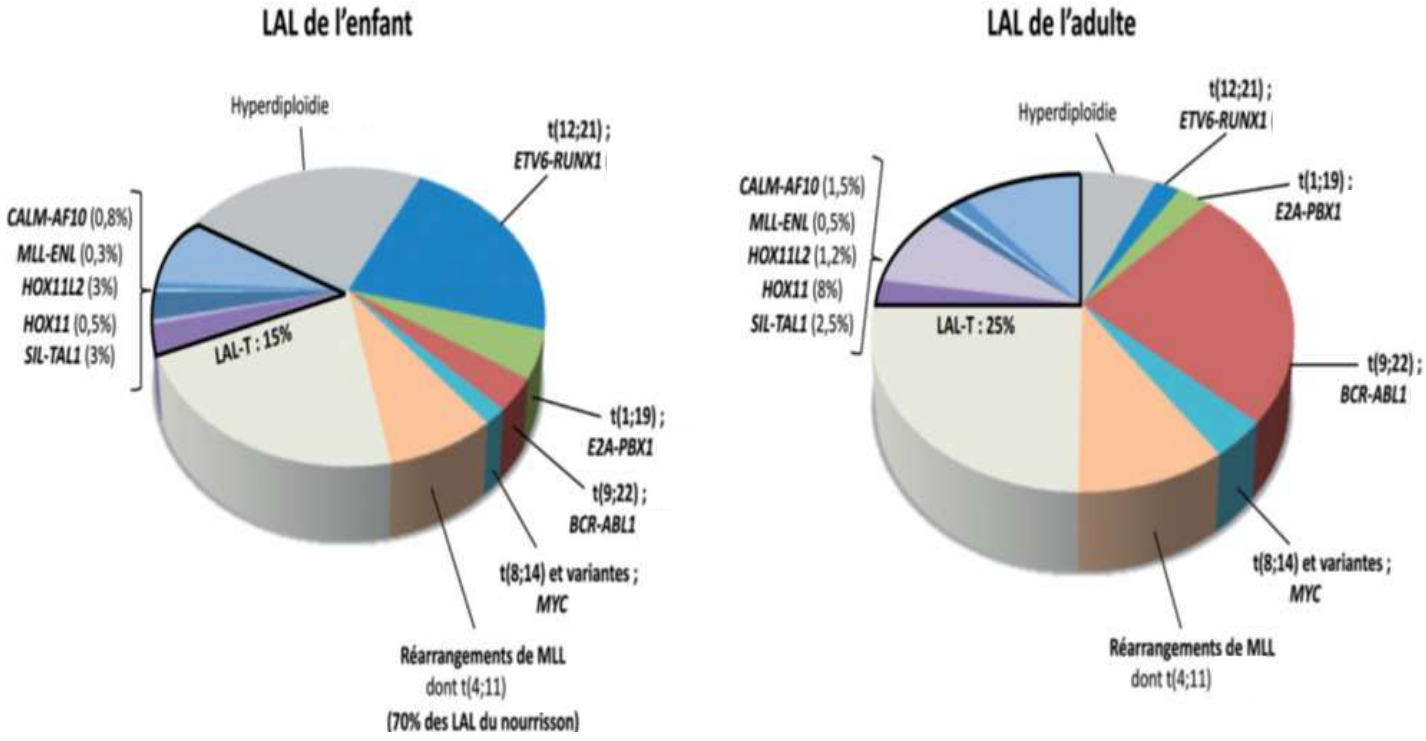

Figure 8: Molecular abnormalities of ALL. Adapted from (Duployeza N and Preudhomme C .2015).

2.6 ALL Treatment

The treatment options of acute lymphoblastic leukemia are dependent on several important factors that should be taken into consideration to establish a treatment plan including cytogenetic abnormalities of the blasts as well as clinical features e.g. the patient's age and involvement of the central nervous system (CNS) (**Pui P et al. 2003**). The main current treatment for ALL is typically long-term chemotherapy (for 2-3 years) and for patients with CNS diseases they are given cranial irradiation and they receive more intense and prolonged chemotherapy.

Treatment of ALL typically takes place in three phases:

2.6.1 Remission induction

Remission induction includes intensive chemotherapy for a month and is intended to eliminate the bulk of disease in bone marrow along with the normal marrow cells return, and the return of blood counts to normal levels. Various combinations of chemotherapy drugs can be used to target multiple major pathways that play an important role in tumorigenesis and progression of cancer. Combinations may include: Prednisolone or Dexamethasone, Cyclophosphamide, Doxorubicin, Vincristine and L-asparaginase. These drug combinations interfere with *PI3K/AKT/mTOR* and extracellular-signal-regulated kinase (*ERK*) signaling pathways (**Hassanein EHM et al. 2020**)**Song P et al. 2015**). The *AKT/mTOR* and *ERK* signaling pathways are involved in autophagy and apoptosis induced by chemotherapy drugs in cancer cells (**Zhang M et al. 2016** and **Song P et al. 2015**).

High risk patients may receive additional Daunorubicin. For BCR-ABL1 ALL and CML patients whose leukemic cells have the Philadelphia (Ph) chromosome, they also receive a tyrosine kinase inhibitor like Imatinib, Dasatinib, Nilotinib and Bosutinib (**Fielding AK. 2015** and **Hochhaus et al. 2020**). For elderly patients, or patients with chronic conditions, medication doses may need to be reduced. In children, about 85 to 90% with AML and 80% with ALL will recover with induction chemotherapy. Whereas, in adults with AML and ALL, about half of them go into remission after induction because leukemic cells may still be hiding somewhere in the body, further treatment is needed.

2.6.2 Intensification (consolidation)

The next phase after remission is the consolidation which often eliminates any remaining disease by using the same combination of chemotherapeutic agents that were used for induction therapy. This phase typically lasts for around 3 months (**Enshaei A et al. 2013**). Usually the drugs are given in high doses so that the treatment is still fairly intense. CNS prophylaxis/treatment is given to eliminate CNS disease. For BCR-ABL1 ALL patients, a tyrosine kinase inhibitor like Imatinib or Dasatinib is also continued for patients. For patients in remission, who are still at high risk for the leukemia relapsing, an allogeneic stem cell transplant (SCT) can be recommended at this time.

Stem cell transplantation (SCT) involves replacing the hematopoietic stem cells in the bone marrow of patients with new healthy blood cells from donor's bone marrow to replace the cells that were killed by chemotherapy.

2.6.3 Maintenance

After consolidation, the patient is generally put on a maintenance chemotherapy program of parenteral Methotrexate (MTX) and oral 6-mercaptopurine (6-MP). Maintenance phase usually last over a period of 2-3 years (**Schmiegelow K et al. 2014**). For BCR-ABL1 ALL patients, a tyrosine kinase inhibitor like Imatinib or Dasatinib is often included as well. CNS prophylaxis continues during maintenance (**Jabbour E et al. 2010**). Careful monitoring of drug toxicities and compliance to drugs is essential for the whole duration of maintenance therapy.

2.6.4 CNS treatment or prophylaxis

CNS prophylaxis is recommended in patients with highly aggressive non-Hodgkin lymphoma (NHLs), such as Burkitt lymphoma and diffuse large B-cell lymphoma (DLBCL) (**Hall K et al. 2018**). Since ALL can spread to the area around the brain and spinal cord (CNS), so central nervous system (CNS) prophylaxis treatment has become an important part of ALL treatment.

The initial CNS prophylaxis consisted of cranio-spinal irradiation. However, due to its toxicity, radiation use has now been reserved for patients at high risk for central nervous system relapse (**Richards S et al. 2013**).

Treatment is given either as prevention, to prevent leukemic cells from spreading to the CNS or to treat the leukemia if it has spread in the CNS. CNS prophylaxis may include Intrathecal chemotherapy, where chemotherapeutic agents like Methotrexate, Cytarabine or Prednisone are injected directly into the cerebrospinal fluid. It may also include high-dose IV Methotrexate, Cytarabine, or other chemotherapeutic drugs and a radiation therapy to the brain and spinal cord (**Qualls D and Abramson J. 2019)**.

2.7 Toxicity of ALL therapy

Chemotherapy has increased the chances of survival in ALL patients, but it also has a host of side effects, such as infection, organ damage, hematological diseases and others (malnutrition, hair loss). Chemotherapeutic agents that suppress immunity increase the risk of bacterial, viral and fungal infections. Infection remains a leading cause of death in ALL children (**Hao K et al. 2020**). Each organ may be affected by ALL treatment; however, the most common complications other

than infection (32.3%) are hepatotoxicity (28.2%), gastrointestinal toxicity (20.4%), central and/or peripheral neuropathy (2.9%) and cardiac dysfunction (2.7%) (**Zawitkowska J et al. 2019**).

2.8 ALL Relapse

Relapse in ALL is the return of ALL in patients who have already undergone treatment and reached complete remission. ALL will return in 15 to 20 percent of the children treated for this disease and have achieved an initial complete remission, while about 50% of adult patients have experienced a relapse (**Cooper S and Brown P. 2015**). Poor prognosis correlates with features such as (National Cancer Institute) NCI- high risk category, early relapse, age >10 years, CNS disease at diagnosis, male gender, T-cell immunophenotype, high risk cytogenetics (**Masurekar A et al. 2014**) and MRD at the end of induction (**Raetz E et al. 2008**).

The main relapses found are listed below, usually they are located at the bone marrow level, but extra medullary forms can be observed at the level of testicles or meninges most often.

2.8.1 Spinal cord relapses

This is the main relapse observed, affecting one in three children and mainly occurring during treatment. Early relapse is a poor prognosis. Bone marrow relapses are primarily treated with allogeneic bone marrow transplantation.

2.8.2 Meningeal relapses

Meningeal relapses are found in 5 to 10% of cases (**Hu S et al. 2020**).Certain karyotypic abnormalities (in particular t (9; 22)), the presence of blasts in the CSF at the initiation of treatment, a high number of WBCs at the time of diagnosis are factors favoring this type of relapse. Meningeal relapse in ALL patients is accompanied often with the following neurological signs:

- Intracranial hypertension
- Facial paralysis (which shows damage to the cranial nerves)
- The presence of blast in the CSF found in control Lumbar Puncture (LP)
- Significant weight gain linked to overeating of central origin.

Regular clinical examinations as well as the control Lumbar Puncture allow early detection of a meningeal relapse of ALL. Consolidation chemotherapy with Methotrexate by intrathecal route will be proposed to treat this type of relapse.

2.8.3 Testicular relapses

This type of relapse is rare and affects 5% of boys. Testicular relapse may occur as mono- or bilateral painless testicular enlargement with infiltration of leukemic lymphoblasts.A puncture or biopsy of the testis can confirm the relapse (**Ceppi F et al. 2014**). These relapses are treated with a new course of chemotherapy as well as testicular irradiation. This irradiation must be bilateral even if only one testicle is affected.

Treatment of relapsed ALL is usually more intense than the newly diagnosed ALL. Recently, new targeted therapies have been tested and found to be important in improving outcomes in patients with relapsed ALL. These targeted therapies include: Novel combinations of chemotherapy drugs, Antibody-targeted therapies like (Blinatumomab and Inotuzumab)and the Chimeric antigen receptor (CAR) T-cell therapy, which involves genetically engineering the patient's own immune cells (T-cells) to target and kill leukemic cells. In addition to the aforementioned targeted therapies, Venetoclax and Navitoclax, which are potent, highly selective and orally bioavailable BCL-XL/BCL2 inhibitors, promote lymphoblastic apoptosis by directly inhibiting their inducible targets, releasing proapoptotic proteins, and triggering mitochondrial outer membrane permeabilization and caspase activation (**Pullarkat VA et al.2021**).

2.9 Prognostic factors in ALL

Prognosis is the predicted outcome of the disease and the chances of recovery (**Lee J and Cho B. 2017**). Several factors determine the prognosis and survival of patients in ALL such as:

- Age of the patient: Younger adults, usually those younger than 50 years of age, have a more favorable prognosis than older adults. Among children ,approximately 85% of patients aged 1 to 18 years with newly diagnosed ALL treated on current regimens are expected to be long-term event-free survivors, with 5-year overall survival rates, reaching more than 90%. More precisely, 98% of children in the age group of 3 to 7 attain a complete remission (**Pieters Ret al. 2016; Vora A et al. 2013** and **Möricke A et al. 2016**).

- Gender: Male gender is associated with poor prognosis compared to girls in ALL (**Li S et al. 2015**).
- WBCs count at presentation: The white blood cell (WBC) count at the time of diagnosis is a prognostic factor for ALL. People with a WBC less than 30,000 for B-cell ALL and less than 100,000 for T-cell ALL tend to have a more favorable prognosis.
- Cancer spread to brain and other body organs: The presence or absence of CNS leukemia at diagnosis has prognostic significance. Patients with ALL who present with CNS disease (CNS3) at diagnosis are at a higher risk of treatment failure and poor prognosis. In addition, patients with ALL that has spread to other organs like liver, spleen and testicles have a less favorable prognosis.
- Morphological, immunological, and genetic Subtypes: High hyperdiploidy generally occurs in cases with clinically favorable prognostic factors and is an independent favorable prognostic factor (**Paulsson K, Johansson B. 2009 and Dastugue N et al. 2013**). Within the hyperdiploid range of 51 to 65 chromosomes, patients with higher modal numbers (58–66) appeared to have a better prognosis. The Ph chromosome (BCR-ABL ALL) was associated with an extremely poor prognosis, and its presence had been considered an indication for allogeneic hematopoietic stem cell transplantation (HSCT) in patients.
- Race and ethnicity: studies showed that survival rates in black and Hispanic children with ALL have been somewhat lower than the rates in white children with ALL (**Bhatia S.2004** and **Kahn J et al. 2018**). The factors associated with race and ethnicity and that influence survival are: ALL subtype, treatment adherence and ancestry-related genomic variations.
- Initial response to the treatment: The prognosis is better if you have no evidence of leukemia 4 to 5 weeks after starting treatment.

3 Signaling in Lymphocyte Activation

3.1 TCR signaling

T cells are a major component of the adaptive immune response, playing a central role in pathogen elimination and tumor surveillance. More than two decades ago, several groups identified the receptor responsible for antigen recognition, the multisubunit T cell receptor (TCR) (**Sharpe M et al. 2015**). T-cell receptors are located only on the cell membrane and are present as two types consisting of two polypeptide chains. In human cells, the most common type of receptor

(95%) is called alpha-beta (αβ) because it consists of two different chains, α (alpha) chain and β (beta) chain. Each chain contains two folded domains, one constant and one variable, and the variable domains of the chains form an antigen-binding site. The least common type is (γδ) gamma-delta receptors (5%), which contain a different set of chains, namely γ (gamma) chain and one δ (delta).

TCR signaling plays a critical role in the lineage specification and development of specialized T cell subsets, including γδ T cells, invariant natural killer T (iNKT) cells and regulatory T (Treg) cells, by promoting a number of signaling cascades that ultimately regulate cytokine production, cell survival, proliferation, and differentiation. The involvement of TCR by stimulatory peptide associated with MHC initiates TCR signals and results in the formation of the immune synapse (IS) between T cells and Antigen Presenting Cells (APCs) (**Huppa J and Davis M. 2003**). T-cell interaction with *APC* also engages stimulatory signaling receptors such as *CD28*, and leads to the employment of *CD8* or *CD4* co receptors that bind to the regions preserved in MHC class I or MHC class II complexes, respectively (**Podojil J and Miller S. 2009**). The intracellular domains of *CD4* and *CD8* bind to *LCK* tyrosine kinase and their co-clustering with the TCR promotes LCK-mediated phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of CD3 subunits (γ, δ, ε and ζ), which then serve as docking sites for Syk-family kinases (**Love PE et al. 2010).**

Phosphorylation of CD3 subunit ITAM tyrosines by LCK allows recruitment of the Syk family kinase, ZAP-70, to the TCR and facilitates activation of ZAP70 by LCK. Activated ZAP-70 then phosphorylates the scaffolding transmembrane adaptor linker for activation of T cells (LAT). LAT is a transmembrane protein with 9 sites of potential tyrosine phosphorylation (**Lo W et al. 2018**). Tyrosine phosphorylation of the C-terminal four sites of LAT leads to the recruitment of adaptor molecules such as growth factor receptor-bound protein 2 (GRB2), GRB2-related adaptor downstream of Shc (GADS), adhesion and degranulation promoting adaptor protein (ADAP) and SH2-domain-containing leukocyte protein of 76 kDa (SLP76). Tyrosine phosphorylation of LAT also recruits and activates the effector signaling molecules including phospholipase C*γ*1 (PLC-γ1), IL-2-inducible T cell kinase (ITK) and Vav Guanine Nucleotide Exchange Factor 1 (VAV1), resulting in the formation of the LAT signalosome (**Gaud G et al. 2018**). Activated PLC-γ1 cleaves phosphatidylinositol biphosphate (PIP2) to the second messengers diacylglycerol (DAG) and inositol triphosphate (IP3), which triggers an increase in intracellular calcium via the release of Ca^{2+} from endoplasmic reticulum storage sites and the influx of extracellular Ca^{2+}.The calcium-bound calmodulin (Ca^{2+}/CaM) activates the protein phosphatase calcineurin by disrupting the

inhibitory effects of calmodulin. Activation of calcineurin leads to the dephosphorylation of its substrates, including NFAT transcription factors (NFAT), allowing their nuclear translocation. Also, DAG activates protein kinase C (PKC), which in turn leads to the nuclear translocation of the transcription factor NFκB (**Figure 9**). Recruitment of GRB2 and VAV1 leads to activation of RAS and ERK–MAPK signaling, resulting in actin polymerization and the activation of the transcription factors FOS, JUN and activator protein 1 (AP-1). These outcomes together with co-stimulatory receptor and cytokine receptor signals, orchestrate multiple T cell responses, including proliferation, migration, cytokine production and effector functions.

Indeed, TCR signal transduction is very complex. There is a described pathway for TCR signals that is not LAT dependent, which is the p38 dependent pathway (**Salvador J et al.2005**). The p 38 dependent pathway includes activation of p38 kinase, which can be phosphorylated directly by ZAP-70. P38-dependent TCR signals seem to favor the mostly Th2 response, characterized by the synthesis of cytokines such as interleukin-4 (IL-4), IL-5, IL-13, and especially IL-10 (**Dodeller F et al. 2006**).

There are also a number of additional molecules that play roles as positive or negative regulators of TCR signaling at three critical nodes centered on LCK, ZAP70 and LAT.

LCK kinase activity is controlled by phosphorylation or dephosphorylation of the key regulatory tyrosines, Y394 (activation) and Y505 (inactivation). CSK promotes phosphorylation of Y505 and exerts negative regulatory control on LCK activity; also, SHP-1 and SHP-2 phosphatase have been reported to dephosphorylate Y394 and inhibit LCK activity and suppress TCR signals (**Pao L et al. 2007)**.

However, the transmembrane tyrosine phosphatase CD45 dephosphorylates pY505 renders the LCK active (**Riley J et al. 2005**). CD45 knockout T cell lines contain Lck that are highly phosphorylated at negative regulatory tyrosines (Y505) and exhibit a drastic reduction in TCR-stimulated phosphotyrosine induction. CD45 knockout mice have few peripheral T cells because TCR signaling during thymic development is impaired (**Palacios E and Weiss A. 2004).**

Likewise, TCR signaling could be regulated at the ZAP70 node by ubiquitin ligase CbI that recruits ubiquitin associating tyrosine phosphatases suppressor of T cell signaling 1 (STS1) and STS2 which were shown to dephosphorylate ZAP70 and inhibit TCR signaling (**Luis BS and Carpino N. 2014** ; **Zhou X et al. 2021**). Mice lacking both STS1 and STS2 are shown to be hyper-responsive to T cell receptor stimulation, resulting in an increase in both cytokine production and susceptibility to autoimmunity (**Zhang J et al. 2015)**.

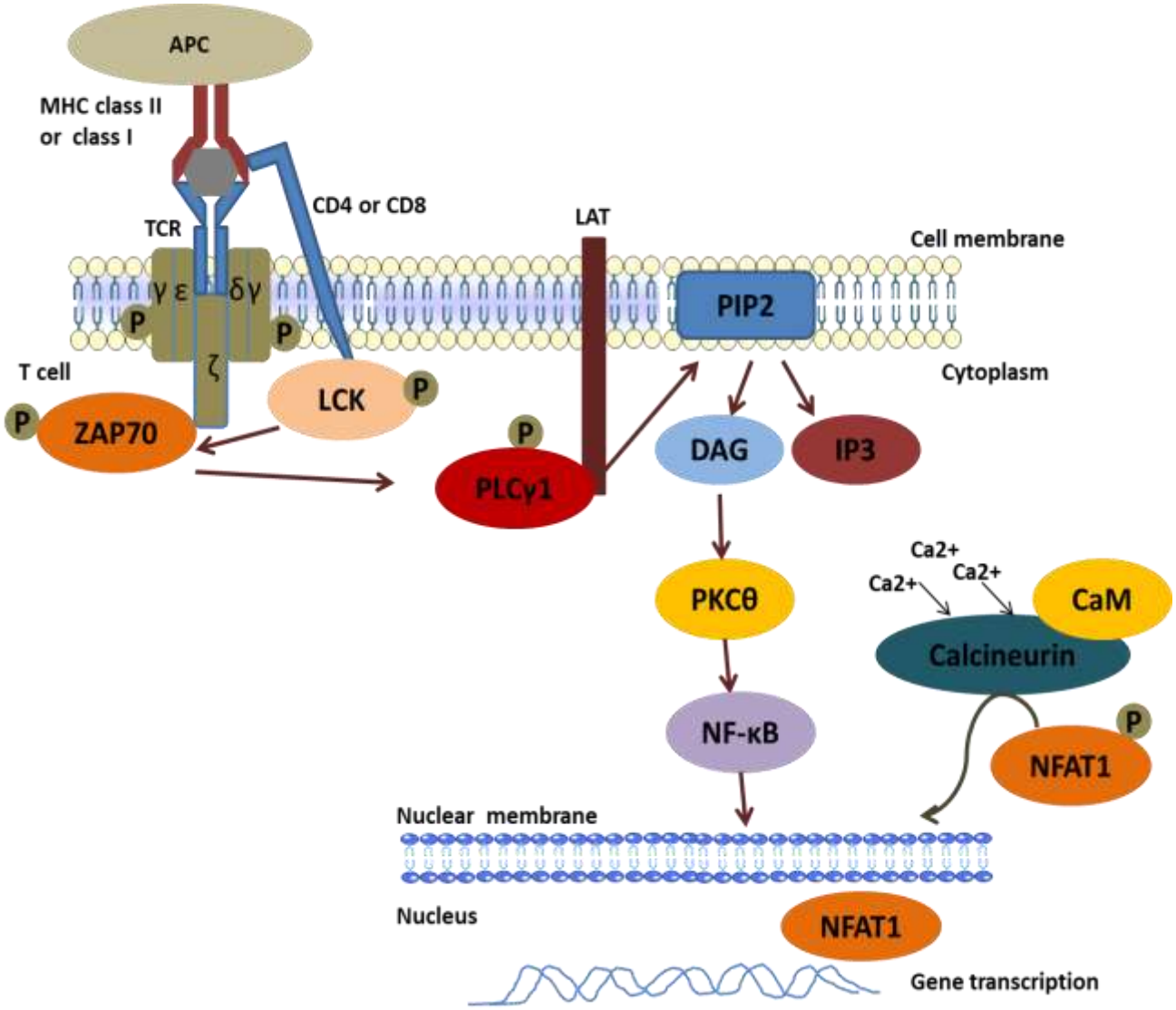

Figure 9: TCR and LCK Signaling pathways. T Cell Receptor (TCR) activation promotes a number of signaling cascades that ultimately determine cell fate through regulating cytokine production, cell survival, proliferation, and differentiation. An early event in TCR activation is phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytosolic side of the TCR/CD3 complex by lymphocyte protein tyrosine kinase (LCK). The CD45 receptor tyrosine phosphatase modulates the phosphorylation and activation of LCK and other SRC family tyrosine kinases. Zeta-chain associated protein kinase (Zap-70) is recruited to the TCR/CD3 complex where it becomes activated. Activated ZAP-70 then phosphorylates the scaffolding transmembrane adaptor linker for activation of T cells (LAT). LAT leads to the recruitment of phospholipase C γ1 (PLC-γ1).Activated PLC-γ1 cleaves phosphatidylinositol biphosphate (PIP2) to diacylglycerol (DAG) and inositol triphosphate (IP3), which triggers an increase in intracellular calcium via the release of Ca2+ from endoplasmic reticulum storage sites and the influx of extracellular Ca2+.The calcium-bound calmodulin (Ca2+/CaM) activates the protein phosphatase calcineurin by disrupting the inhibitory effects of calmodulin. Activation of calcineurin leads to the dephosphorylation of nuclear factor of activation T cell (NFAT), allowing it to enter the nucleus. Also, DAG activates protein kinase C (PKC), which in turn leads to the nuclear translocation of the transcription factor NFκB. The activity of these transcription factors results in T cell proliferation, migration, cytokine production and effector functions. Drawn with information from (Gaud G et al. 2018).

3.2 BCR Signaling

B cells mediate the humoral immune response, which is a component of the adaptive immune system activity. B cells have several receptors that transduce external signals and influence the fate of the B cell. However, the principal signaling pathway in B cell activity is the B Cell Antigen-Receptor (BCR) pathway (**Figure 10**), because it facilitates a variety of signaling results such as survival, proliferation, differentiation and apoptosis. Also, any disruption in the regulation of BCR signaling leads to autoimmunity and cancer.

The B-cell Receptor Complex is composed of a membrane immunoglobulin (IgM) disulfide-bonded to the heterodimers CD79a and CD79b (**Woyach J et al. 2012**). IgM is composed of two identical heavy chains and two identical light chains. The cytoplasmic regions of CD79a and CD79b are called ITAMs (immunoreceptor tyrosine-based activation motif) (**Packard T and Cambier J. 2013**).

Upon engagement of the IgM molecules, the cytoplasmic tails of CD79a and b are phosphorylated by LYN, a SRC family kinase. This leads to binding and phosphorylation of Syk. Syk phosphorylates Vav-1, in addition to BTK and PLCγ2, which are assembled by BLNK (SLP-65) .Phospholipase C-γ2 (PLC-γ2) cleaves phosphatidylinositol 4,5-biphosphate (PIP2) to form diacylglycerol (DAG) and inositol triphosphate (IP3) (**Kadamur G and Ross E. 2013**) which triggers an increase in intracellular calcium via the release of Ca^{2+} from endoplasmic reticulum storage sites and the influx of extracellular Ca^{2+} (**Mattson M and Chan S. 2003**). Ca^{2+} and DAG activate protein kinase C (PKCβ) which in turn leads the nuclear translocation of the transcription factor NFκB, that plays a central role in regulating many aspects of B cell differentiation and function (**Lim P et al. 2015**). Cytosolic Ca2+ facilitates the activation of calcineurin by calmodulin, which directly activates NFAT. NFAT involved in the development of mature B cells regulates the expression of many genes **(Scharenberg A et al. 2007).** PLCγ2 also activates the mitogen-activated protein kinase (MAPK) pathways, including (ERK1/2), (JNK) and p38 MAPK.Vav1 recruits PI3K, which in turn activates AKT, leading to increased cell survival and proliferation. Other membrane proteins, such as CD19, complex with LYN and assist in B cell activation by lowering the receptor's threshold for antigen stimulation (**Merolle M et al. 2018**).

BCR signaling is balanced by positive and negative regulators at the level of Syk, LYN, PI3K, and ERK activity (**Packard T and Cambier J. 2013**). For example, the phosphatase SHP1 downregulates Syk activity, Csk downregulates LYN, PTEN downregulates PI3K, and DUSP6 downregulates ERK. While The CD19/CD81 upregulates PI3K.

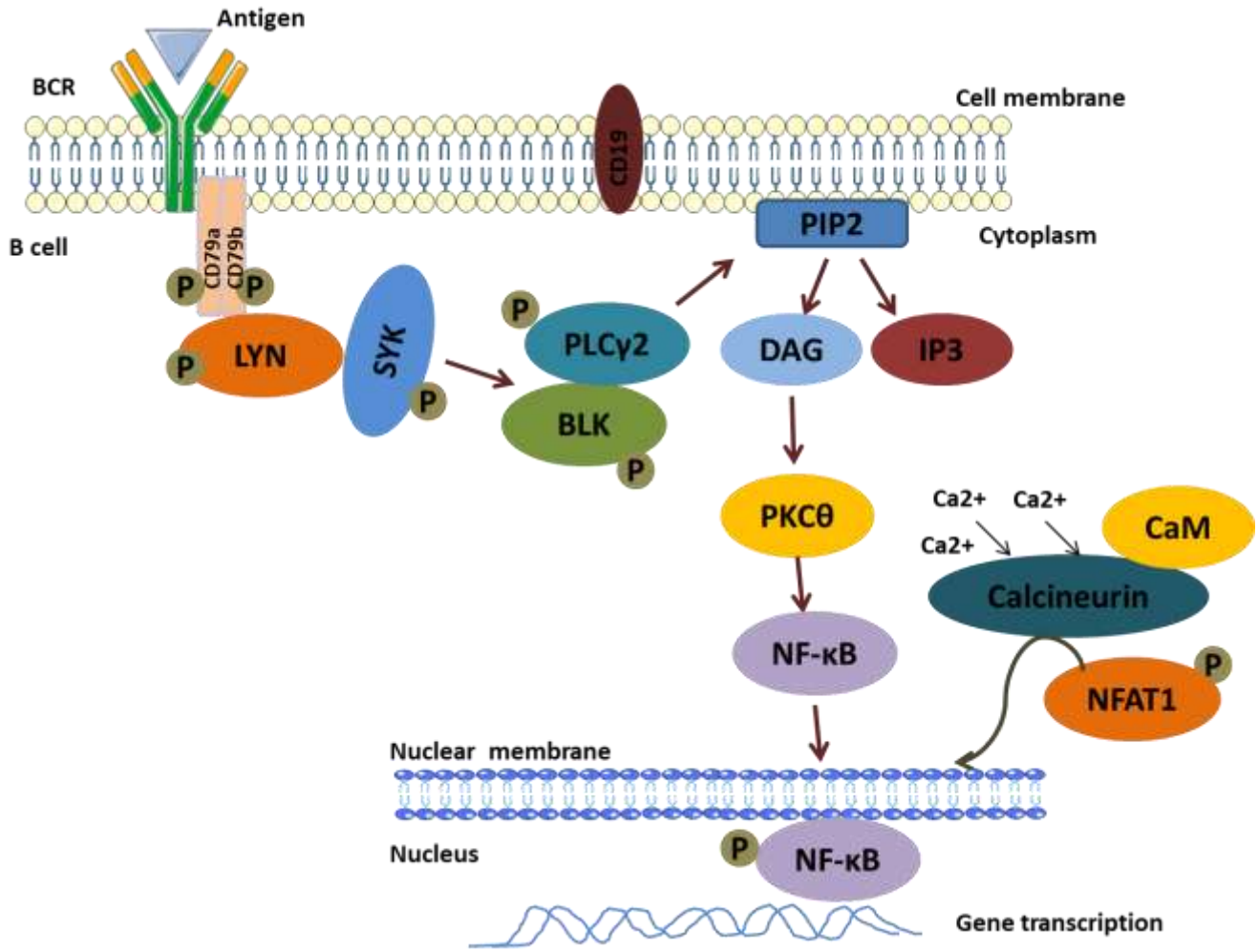

Figure 10: BCR and LYN signaling pathways. The B-cell receptor (BCR) signaling pathway in normal B-cells induces apoptosis, proliferation, migration or survival. Antigen (Ag) binding to surface immunoglobulins (sIg) triggers BCR (B-Cell Receptor) activation by inducing its translocation to lipid rafts, where the co-stimulating immunoglobulins heterodimer (CD79a/b) is phosphorylated by Syk and LYN kinases. This leads to binding and phosphorylation of Syk. Syk phosphorylates BTK and PLCγ2, which are assembled by BLNK (SLP-65) .Phospholipase C-γ2 (PLC-γ2) cleaves phosphatidylinositol 4,5-biphosphate (PIP2) to form diacylglycerol (DAG) and inositol triphosphate (IP3) which triggers an increase in intracellular calcium via the release of Ca^{2+} from endoplasmic reticulum storage sites and the influx of extracellular Ca^{2+} . Cytosolic Ca2+ facilitates the activation of calcineurin by calmodulin, which triggers the nuclear translocation of Nuclear Factor of Activated T-cells (NFAT). Ca^{2+} and DAG activate protein kinase C (PKCβ).Downstream signaling of PKC, leads the nuclear translocation of the transcription factor Nuclear Factor-kB (NFκB) that plays a central role in regulating many aspects of B cell differentiation and function. Adapted from (Bojarczuk K et al. 2015).

4 SRC family kinase

The SRC family kinase is a family of non-receptor tyrosine kinases; these protein kinases are present in essentially all metazoan cells. While their activation is critical for generating an appropriate cellular response to external stimuli, many members of SRC family kinase have also been implicated as being hyperactive in various diseases including the neoplastic transformation of

cancer/leukemia **(Warmuth M et al. 2003)**. These prompted scientists to target these kinases to develop therapeutic inhibitors to treat Human cancer and leukemia (**Warmuth M et al. 2003**).

The prototype member of the SRC family protein tyrosine kinases was first identified as the transforming protein (v-SRC) of the oncogenic retrovirus, Rous sarcoma virus (RSV) (**Simatou A et al. 2020**). SRC family kinases are regulatory proteins; they have been heavily implicated in the regulation of metabolism, viability, proliferation, differentiation and migration within many different cell lineages (**Frame MC et al. 2002**). This wide range of activities is a result of the ability of these kinases to associate with various classes of cellular receptors and many distinct cellular targets.

In addition, these members can be subdivided into three groups based on their general pattern of expression, those that are SRC related (SRC, Yes, Fyn and Fgr), those that are LYN related (LYN, Hck, LCK and Blk) and those that are Frk related (Brk, Frk and Srm) kinases (**Table 5**).

The members of first group, SRC, Fyn, Yes and Fgr are expressed in most tissues. Although SRC is expressed everywhere, however, platelets, neurons, and bone cells express a 5- to 20-fold higher protein level than most other cells (**Roskoski R. 2004**).

The members of second group, Blk, Hck, LCK, and LYN, are found primarily in hematopoietic cells (**Wang J and Zhuang S. 2017)**. Both LCK and LYN have also been detected in brain, LCK expression has been detected in distinct brain regions including the hippocampus and cerebellum, LYN appeared in both embryonic and adult brain. Frk-related kinases are expressed predominantly in epithelial-derived cells (**Ahluwalia M et al. 2010; Ogunbolude Y et al. 2017**).

SRC family member	Pattern of expression	Oncogenic forms
Blk	B cells	Overexpressed in B-cell acute lymphocytic leukemia
Fgr	Myeloid cells, B cells	Overexpressed in some leukemias and lymphomas
Fyn	Ubiquitous	-
Hck	Myeloid cells	-
Lck	T cells, NK cells, brain	Overexpressed in T-cell acute lymphocytic leukemia
Lyn	B cells , brain, Myeloid cells,	Overexpressed in B-cell acute lymphocytic leukemia
Src	Ubiquitous	Overexpressed in mammary ,pancreatic and other cancers
Yes	Ubiquitous	Highly expressed in colon, malignant melanoma and other cancers.

Table 5: Characteristics of SRC family kinases

4.1 Structural Domains of SRC Kinases

SRC family kinase members are 52–62 kDa proteins; they share the same domain arrangement: a large catalytic C-terminal domain, Src 1 homology (SH1), Src 2 homology (SH2), Src 3 homology (SH3) domains preceded by a ~80 residue region of low conservation called the Unique (U) domain and a SH4 region at the N terminus. While the structured domains (SH3, SH2 and SH1) are highly homologous, the SH4 domains and unique domains share very little sequence similarity among the family (**Figure 11**).

SH4 domain

The SH4 domain spans the first 10–15 residues and drives membrane association via three distinct features: N-terminal myristoylation (in all SFKs), an SFK-dependent number of palmitoyl groups (in all but SRC and BLK), and a lysine rich stretch of amino acids that augments lipid binding and directs SFKs to negatively charged membranes.

Unique domain

The unique domain (UD) of each individual SFK member is well conserved between different organisms suggesting a more specific role than that of a simple spacer. It has been proposed to be important for mediating interactions with receptors or proteins that are specific for each family member. For example, the unique domain of *LCK* is known to mediate association with the cytoplasmic tails of T cells coreceptors of: *CD4* and *CD8* (**Kim et al. 2003).**

SH3 domain

The SH3 domain directs specific association with proline rich motifs related to the PXXP consensus, it is important for intra- as well as intermolecular interactions that regulate SRC catalytic activity, SRC localization, and recruitment of substrates

SH2 domain

The SH2 domain controls the group of proteins interacting with SRC tyrosine kinases, it allows proteins containing those domains to dock to phosphorylated tyrosine residues on other proteins.

SH1 domain

The SH1 is the last domain; it is responsible for the enzymatic activity. In case of c-SRC, SH1 domain possesses the classical kinase activation loop tyrosine 419 (Tyrosine site Y 419) which is phosphorylated in the active state. Also it possesses a short C-terminal tail, which bears an auto-inhibitory phosphorylation site (Tyrosine site Y 527) that is phosphorylated by Csk in the inactive state (**Byeon SE et al. 2012)**.

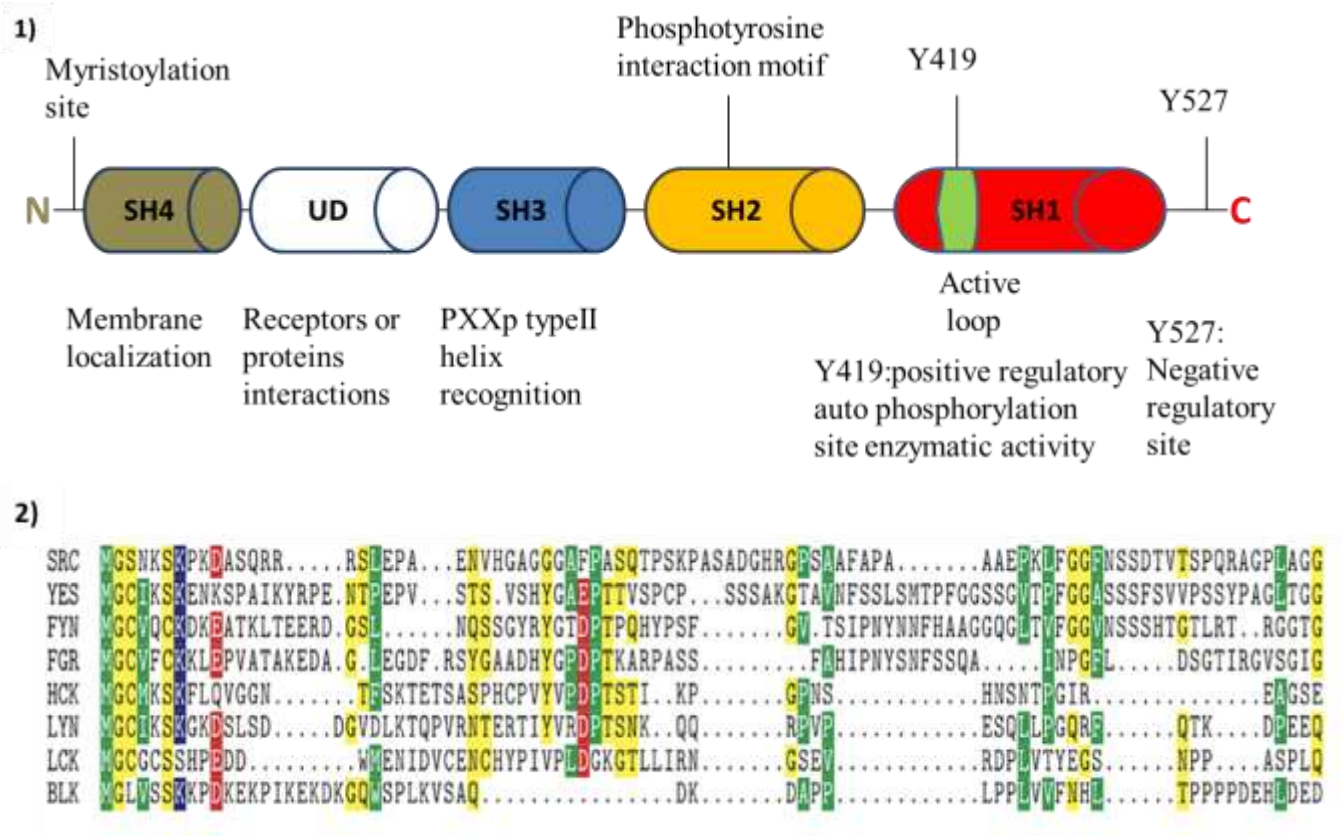

Figure 11: The domain structure of SRC family kinases and the sequence comparison of SFK. (1)The SRC kinase architecture consists of 5 domains: the SH4 domain and unique region, which varies among family members, followed by the SH3, SH2, and tyrosine kinase domains. The approximate extent of each domain is indicated, with the SH4, UD, SH3, SH2, and SH1 colored green, white, blue, yellow and red purple, respectively. SH4 domain is important for membrane localization. The unique domain has been proposed to be important for mediating interactions with receptors or proteins that are specific for each family member. SH3 which directs specific association with proline rich motifs related to the PXXP.SH2 controls the group of proteins interacting with SRC tyrosine kinases.SH1 which is responsible for the enzymatic activity .The activation loop of the kinase domain, the activating (Tyr 419) and autoinhibitory (Tyr 527) phosphorylation sites are indicated. N: N-terminal sequence. C: C-terminal sequence. (2) Low sequence conservation of human SFKs in the SH4-U region. Adapted from (Pond MP et al.2020)

4.2 SRC family members

4.2.1 **LCK**

The human *LCK* gene is located on chromosome lq35-34.3 and has 12 exons distributed across ~14kb of genomic DNA. The expression of the gene is regulated by 2 promoters: a proximal and a distal promoter. The *LCK* gene encodes p56 (LCK) protein. LCK (leukocyte-specific tyrosine kinase) is a 56 kDa protein found mainly in hematopoietic cells. It is most commonly expressed in T cells, NK T cells and to a lesser extent in B cells. LCK expressed on the cell membrane of T cells, plays a major role in signaling of the T-cell antigen receptor (TCR). LCK activation is required for the proliferation and differentiation of T cells.

LCK is also essential for thymocyte development (**Rudd ML et al. 2006**) and may play a role in mitochondrial apoptosis independent of its principle function in TCR signaling (**Samraj A et al. 2006**).

4.2.1.1 LCK's protein structure and activation

The N-terminus is a short sequence for lipid attachment mediated by myristoylation and palmitylation, which are involved in the distribution and localization of kinases to the membrane. In the inactive state, closed conformation of LCK is stabilized when the C-terminal tail is phosphorylated at the tyrosine site 505 (equivalent to tyrosine site Y 530 of c-SRC) by C-terminal SRC kinase (Csk) (**Okada M. 2012**). The active conformation is promoted by phosphorylation of a tyrosine (Y394 in LCK) in the activation loop of the kinase domain (equivalent to tyrosine site Y 419 in c-SRC) which leads to an increase in LCK's catalytic activity (**Figure 12**) (**Nika K et al. 2010**). This activation of LCK in fibroblasts and in T-lymphocytes (in vitro) is due to CD4 T-cell surface antigen which physically binds to LCK tyrosine protein kinase and mediates its rapid enzymatic activation, after antibody-mediated cross-linking.

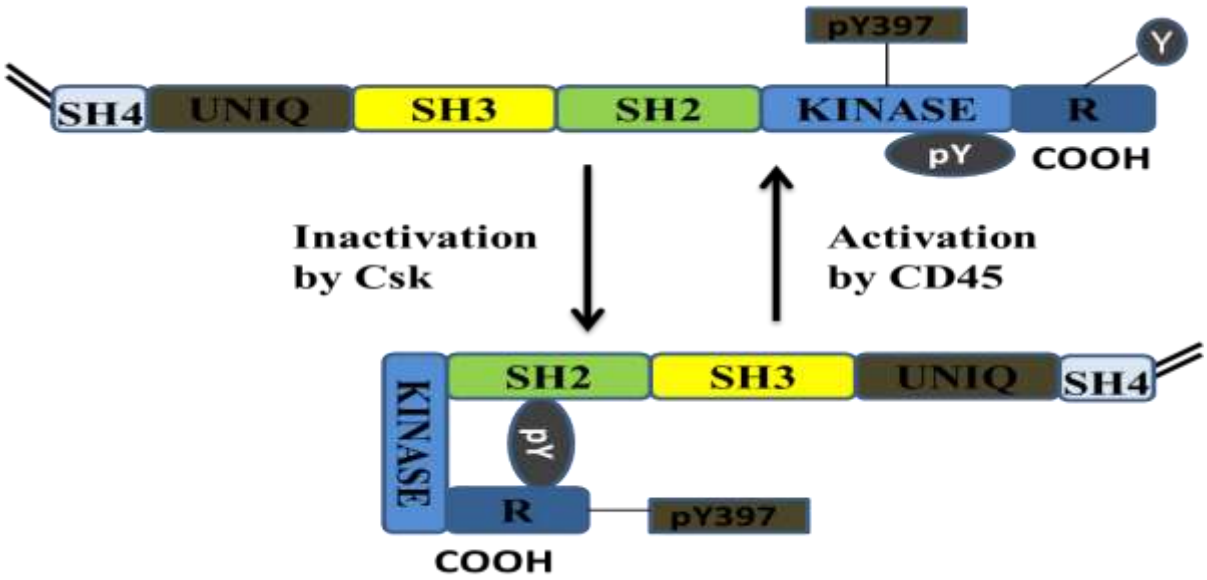

Figure 12: Representation of LCK structure and regulation of its kinase activity. LCK conformation and regulation of LCK activity. Phosphorylation of the inhibitory tyrosine (Y505) by Csk results in a close/inactive conformation .Auto- trans-phosphorylation of Y394 results in an open/active conformation. Dephosphorylation of Y394 by phosphatases reverts active LCK back to the primed conformation. Adapted from (Bommhardt U et al. 2019)

4.2.1.2 LCK expression in cancer and leukemia

From many years ago, LCK was overexpressed in lymphocytic leukemia of the B-cell lineage such as B-cell precursor form of ALL (BCP-ALL) (**Cazzaniga V et al. 2015**) and in AML (**Marhäll A et al. 2017**); in addition to be overexpressed, LCK was found to be hyperactivated. Additionally, LCK expression was detected in a number of solid cancers including breast cancer (**Vahedi S et al. 2015**) colorectal cancer (**Clarke CN et al. 2017)** and lung carcinoma (**Meng Y et al. 2021**). Recently, phosphoproteomic profiling analyses of newly diagnosed pediatric T-ALL patients were performed by using reverse-phase protein arrays (RPPA) and it has shown that the lymphocyte cell-specific protein-tyrosine kinase (LCK) was hyperactivated in these patients. Also, LCK was identified as aberrantly active in prednisone poor responders (PPR) patients and that it could be responsible for the glucocorticoid resistance observed in some cases of T-ALL (**Serafin V et al. 2017**). These observations have led to the hypothesis that LCK may have cancer promoting functions and hence may represent a potential diagnostic biomarker and therapeutic target for cancers.

Consequently current therapeutic efforts have focused on targeting LCK activity using SRC family kinase inhibitors like Dasatinib and Saracatinib. The results have shown that these inhibitors diminish LCK activation and impair maintenance of human T-ALL (**Buffière A et al. 2018**). Moreover, the results showed that the combination of Dasatinib with Dexamethasone reveals synergistic effects on resistant T-ALL cells (**Shi Y et al. 2020**).

The inhibition of LCK, by preventing its phosphorylation, is an important strategy for the treatment of malignant hematopoiesis such as T-ALL, particularly with the use of Bosutinib, Dasatinib or Saracatinib, which affect proliferation of leukemic cells (**Serafin V et al. 2017** and **Buffière A et al. 2018)**. However, no work has been performed to examine the effect of HSP90 inhibitors on the inhibition of SRC kinases and on the treatment of T-ALL cells.

4.2.2 LYN

The abbreviation **LYN** is derived from **LCK**/**Yes** novel tyrosine kinase. LYN is one of the members of the SRC kinase family of non-receptor protein tyrosine kinases, which is mainly expressed in hematopoietic cells (Erythroid/Myeloid and B lymphoid origin) (**Ingley E. 2012**). It has also been detected in neural tissues, adipose tissue, liver, prostate and colon cells (**Goldenberg-Furmanov M et al. 2004** and **Bates R et al. 2001**). LYN was involved in the transmission of signals from a

number of receptors such as Epo, c-Kit, B cell antigen receptor (BCR) (**Lennartsson J et al. 2005**), CD19, CD40 and c-Mpl receptors (**Bates R et al. 2001**). LYN also has been emerged as a key enzyme involved in the regulation of B cell activation. It is a protein that is encoded in humans by the *LYN* gene, this gene is localized on the human chromosome 8q13.

4.2.2.1 LYN's protein structure and activation

LYN has two splice variants (via exon 2) that result in the generation of *p53* and *p56* kDa protein isoforms, designated as LYN A (p56) and LYN B (p53), which differ by a 20 amino acid region in the SH4 domain that encompasses a pY motif (pY32) (**Ingley E. 2012**). As already mentioned LYN structure is very similar to other SRC kinase family members. Whilst this N terminus of each member is unique, the SRC family shares significant homology in the kinase domain, as well as the SH2/SH3 protein interaction domains. LYN is regulated by protein interactions through its SH2/SH3 domains as well as via Tyrosine phosphorylation. In the inactive state, the C-terminal tail is phosphorylated at the tyrosine site 508(equivalent to tyrosine site 527 of c-SRC) in LYN, by C-terminal SRC kinase (Csk) (**Ingley E et al. 2008**). In contrast, in the active state the C-terminal tyrosine (Tyr 508) is dephosphorylated by phosphatases such as CD45 and SHP-2 (**Masato Okada. 2012**). Then, LYN can trans-auto phosphorylate within the activation loop, tyrosine site Y397 (equivalent to tyrosine site Y 419 of c-SRC) to generate a very active enzyme (**Xu Y et al. 2005)**.

4.2.2.2 LYN in leukemia

Beside its implication in the development of some solid cancers, evidence of a strong role for *LYN* is increasing in several types of leukemia and lymphoma. In chronic lymphocytic leukemia (CLL) cells, LYN was aberrantly expressed and highly activated (**Kohlhas V et al. 2020**). Many substrates of LYN were inordinately activated and they supported the aggressiveness and apoptosis resistance of CLL cells.

In acute myeloid leukemia, Lyn was consistently expressed at a high level and constitutively activated. It was found important for survival and proliferation of AML cells. Accordingly, the inhibition of Lyn by the SFK inhibitor (PP2) and the specific down regulation of *LYN* expression by small interfering RNA (siRNA) inhibited mTOR pathway and induced apoptosis (in vitro) (**Dos Santos C et al. 2007)**.

In chronic myelogenous leukemia, LYN kinase was highly overexpressed and activated. It plays a role in CML cells growth and survival. Indeed, the inhibition of LYN kinase by a SRC inhibitor (GP-76030) reduced proliferation and enhanced apoptosis of CML cells (in vitro) (**Donato NJ et al. 2003**).

In Ph (+) B-cell acute lymphoblastic leukemia, LYN is involved in BCR-ABL-induced B-lymphoid leukemogenesis. It is important for survival and proliferation of B-ALL cells. Indeed, the inhibition of SRC kinase by CGP (SFK inhibitor) impaired the proliferation of B-lymphoid cells expressing BCR-ABL in vitro and prolonged survival of mice with B-ALL (in vivo) **(Hu Y et al. 2004)**.

Back to our topic, since LYN appears to have important role in leukemogenesis especially in B-ALL, targeting and inhibition of LYN by preventing its phosphorylation, could be a promising treatment of this disease. Therefore several studies have focused on targeting SRC kinase (LYN) activity using kinase inhibitors like Dasatinib and they found that inhibition of SRC kinase results in long-term survival of mice with B-cell acute lymphoblastic leukemia, and that continuous administration of Dasatinib could prevent ALL stem cells from developing into fatal ALL (**Hu Y et al. 2006** and **Li S. 2007**). Also, the kinase inhibitor CGP76030 and Imatinib impaired the proliferation of B-lymphoid cells expressing Bcr-Abl in vitro and prolonged survival of mice with B-ALL, by targeting important kinases in the BCR pathway like LYN, HCK and FGR (**Hu Y et al. 2004**). So targeting LYN in B-ALL, by Imatinib and other tyrosine kinase inhibitors could be a good choice in treating these diseases.

4.3 SRC TK inhibitors

Given the crucial role of SRC in tumor development and extensive preclinical evidence of metastasis suppression by SRC inhibition, several clinically applicable small molecule *SRC* inhibitors have been developed and are undergoing clinical testing, particularly for metastatic diseases (**Kim L et al. 2009**). There are two main categories of SRC inhibitors currently being developed: SH2/SH3 blocking inhibitors and ATP-competitive kinase inhibitors.

SH2/SH3 blocking inhibitors such as UCS15A, AP22408, and KX239, prevent the SH domain mediated interactions and prevent SRC protein-protein interactions. These inhibitors have demonstrated poor transport and uptake properties and only inhibit a subset of *SRC* protein interactions, limiting the clinical efficacy of this class of SRC inhibitors.

ATP competitive SRC kinase inhibitors target the active site and prevent ATP from binding and initiating the phosphotransferase activity of the enzyme. There is significant homology in the structure and sequence of ATP-binding domains of kinases, which has made the narrowing of ATP competitors' specificity difficult.

However, several ATP competitive inhibitors are currently under various stages of investigation as potential therapies for cancer. PP1 and PP2 were among the first SRC kinase inhibitors utilized to study the role of SRC activity in cellular events. The use of PP2 has shown that inhibition of SRC results in a loss of downstream SRC signaling pathways and elicits anti-tumorigenic phenotypes (**Chen T et al. 2006**). Since it was discovered that inhibition of SRC can lead to anti-tumorigenic effects in cancer models several ATP-competitive SRC kinase inhibitors such as Dasatinib, Saracatinib and Bosutinib have been synthesized and studied.

Dasatinib is a tyrosine kinase inhibitor of ABL, SRC and other SFKs. It is approved for the treatment of certain types of leukemia, breast cancer, prostate cancer and other cancers (**Brave M et al. 2008; Finn R et al. 2006** and **Koreckij T et al. 2009**). Saracatinib is an inhibitor of SFKs, EGFR, c-Kit, EphA2 and the SRC-regulator Csk. It showed anti-proliferative activity and anti-invasive activity in vitro, in several cancer cell lines, including breast, prostate, and colon cancer, and in vivo it showed anti-proliferative activity in leukemia cells (**Green T et al. 2009; Rikiishi H. 2012; Chang Y et al. 2008 and Buffière A et al. 2018**). Bosutinib has demonstrated activity as an inhibitor of SRC, SFKs and BCR-ABL in leukemia and lymphoma cell lines. It also showed promising efficacy in prolonging time to progression in locally advanced or metastatic breast cancer patients (**Campone M et al. 2012** and **Puttini M et al. 2006**). However, despite the important therapeutic activity and the high response rate of SRC inhibitors in various types of cancer, adverse events and drug resistance are two major issues that can affect patients' quality of life and response to drugs.

5 Heat Shock Proteins (HSPs)

Heat shock proteins (HSPs) are a family of proteins produced by cells exposed to stressful conditions like heat, cold, UV light (**Hang K et al. 2018***),* during wound healing or tissue remodeling and bacterial or viral infections (**Xue J et al. 2016**). Heat shock proteins (HSPs) have appeared in all organisms, from humans to bacteria. HSPs have important chaperone functions in maintaining cell homeostasis and cellular growth. They prevent the proteins aggregation in folding and unfolding of protein, they are involved in the transfer of cellular proteins into subcellular

compartments, and they have a regulatory function in cell cycle control, signal transduction and in the protection of cells against stress or apoptosis. HSPs have essential anti-apoptotic properties; they block both the intrinsic and the extrinsic apoptotic pathways by interacting with essential proteins necessary for modulation of signal pathways and for controlling the release of apoptogenic molecules (**Mjahed H et al. 2012**). More recently, it has been found HSPs to be essential for antigen presentation with the role of chaperoning and accompanying antigenic peptides to the major histocompatibility complexes (MHC) class I and II molecules (**Li Z et al. 2002**). In addition, extracellular HSPs can stimulate professional antigen presenting cells for the immune system, such as macrophages and dendritic cells (**Li Z and Srivastava P. 2004)**.

Heat shock proteins (HSPs) are so called because they were first observed in response to hyperthermia. They constitute a large family of proteins classified based on their molecular weight, such as HSP110 (105-110 kDa), HSP90 (85-90 kDa), HSP70 (68-73 kDa), HSP60 (57-69 kDa) and small HSPs (12-43 kDa)**.**The small 8-kilodalton protein ubiquitin, which marks proteins for degradation, also has features of a heat shock protein.

Table 6 lists common *HSPs* and summarizes their characteristics including (a) name, (b) subcellular localization, (c) known function, (d) chromosome assignment, (e) and brief comments.

Heat-shock protein	Other names	Subcellular localization	Known functions	Comments
HSP27	**HSPB1, HSP25**	**Cytosol**	**Antiapoptotic, cytoprotection**	**Heat-inducible, chaperone activity is independent of ATP; high expression correlated**

HSP60	**HSPD1, HSP65 CPN60**	**Mitochondria**	**Cytoprotection; macrophage activator possibly through Toll like receptors**	**Functions along with co-chaperone HSP10; linked with autoimmunity such as rheumatoid arthritis**
HSP70	**HSP70s ,DnaK**	**Cytosol/ nucleus**	**Cytoprotection and antiapoptotic, HSP70-2 implicated in spermatogenesis**	**Strongly inducible by heat shock; known as the inducible form of HSP70**
HSP90α	**HSP90, HSP86-1, HSP89, HSP4**	**Cytosol**	**Protein folding, peptide chaperone, cytoprotection, intracellular signaling (e.g. steroid receptor), cell-cycle control and buffering of harmful mutations**	**Small molecule inhibitor such as Geldanamycin derivatives currently under phase I trial as anticancer agents**
HSP90β	**HSP84-1, HSP84, Hsc90**	**Cytosol**	**Major cytosol chaperone; protein folding; cytoprotection; intracellular signaling (e.g. steroid receptor); cell-cycle control; and buffering of harmful mutations**	**Constitutively expressed; inhibitors such as Geldanamycin derivatives currently under phase I trial as anticancer agent**
HSP110	**APG-2, HSP110, HSPa4, HSP105α, and HSP105β**	**Cytosol/ nucleus**	**Binds to Hsc70 to form high-molecular-weight complex; involved in protein folding, thermotolerance, and embryogenesis**	**Two isoforms in mice; heat inducible; also induced by human papilloma virus oncoprotein E7**

Table 6: Common HSPs and their characteristics

5.1 HSP27

Heat shock protein 27 (HSP27) also known as heat shock protein beta-1 (HSPB1), is a chaperone of the sHSP (small heat shock protein) group among α-crystallin, HSP20, and others. It is a 27-kDa protein ubiquitously expressed by many cell types specially the vascular cells.

5.1.1 Genes

HSP27 is a protein that in humans is encoded by the *HSPB1* gene.

5.1.2 Proteins

The primary structure of HSP27 is highly homologous to other members of the small HSP family, containing the conserved α-crystallin domain and differing in the C- and N-terminal regions.
HSP27 regulates the activity of multiple kinases and activates and inhibits multiple signaling pathways. These pathways include MAPK signaling pathway, calcium signaling pathway Wnt signaling pathway, insulin signaling pathway, ERB-B signaling pathway, TGF-B signaling pathway, JAK-STAT signaling pathway and integrin signaling pathway (**Cheng J et al. 2015**).

5.1.3 Expression

HSP27 is expressed in all human tissues, including astrocytes and primary neuronal cells but mainly in skeletal, smooth and cardiac muscles. It is located mainly in the cytosol, endoplasmic reticulum, and nucleus (**Wang X et al. 2014**).

5.1.1 Physiological roles of HSP27 and its genetic deletion

Beside the main function of HSP27, which is to provide thermotolerance in vivo, cytoprotection, and support of cell survival under stress conditions, HSP27 has additional roles. It is involved in embryogenesis, cardioprotection, modulation of inflammation and regulation of apoptosis (**Ferns G et al. 2006**). It also plays a role in the fine-tuning of terminal erythroid differentiation through regulation of GATA-1 content and activity (**De Thonel A et al. 2010**).
The depletion of HSP27 has been shown to increase the activation of caspase-3, which sensitizes cells to cell death and promotes apoptosis. Also, the depletion of *HSP27* was associated with several diseases in human, including cardia bifida, neuronal disorders and immunity **(Gibert B et al. 2012).**

5.1.2 HSP27 in cancer

High levels of HSP27 have been reported in various types of cancer, such as breast cancer, endometrial cancer, and leukemia. The tumorigenic potential of HSP27 has also been observed in

experimental models. Furthermore, high expression of this chaperone has been shown to correlate with drug resistance, metastasis and poor patient outcome, which emphasizes its properties in cancer therapy **(Wang X et al. 2014**).

5.2 HSP60

Alternate Names: Heat shock protein 60, HSP-60, P60 lymphocyte protein, CPN60, HSPD1.

HSP60 is a family of heat shock proteins originally sorted by their 60kDa molecular mass. HSP60 family members play essential roles in recovery of denatured proteins under stress conditions and also in protein synthesis during cell growth and survival.

5.2.1 Genes

HSP60 proteins are encoded by *HSPD1* and *HSPE1* genes (**Bross P et al. 2007**).

5.2.2 Proteins

HSP60 proteins have double-ring-like structures and capture denatured substrate proteins in its central cavity. HSP60 regulates the activity of multiple kinases and activates and inhibits multiple signaling pathways. These pathways include NFκB signaling pathway, p38 MAPK pathway and insulin-like growth factor-1 (IGF-1) signaling **(Zhang D et al. 2020**).

5.2.3 Expression

HSP60 is expressed in the reproductive system, particularly in testicles and in the non-reproductive system mainly in the kidneys, heart, cerebellum, liver, lung, and spleen. Although HSP60 is abundant in the mitochondria, its expression can be found in both the cytosol and mitochondria under physiological conditions

5.2.4 Physiological roles of HSP60 and its genetic deletion

HSP60 was found to have both immune-regulatory and inflammatory properties placing it as a dominant antigen recognized during infections (**Coelho V and Faria A**.**2012**), but with potentially harmful effects as well. Indeed, various studies have linked HSP60 to diabetes, stress response, and certain types of immunological disorders. In addition, it was

found that HSP60 plays an essential role in maintaining normal cardiac morphology and function by regulating mitochondrial protein homeostasis and mitochondrial function.

Knockdown of *HSP60* in vivo (mice) and in vitro, leads to decreased IL-1β production during Japanese encephalitis virus (JEV) infection, which eventually leads to decreased inflammation and increased survival of JEV-infected mice (**Swaroop S et al.2018).** Moreover, by generating a heart-specific *HSP60* knockout mouse model, it has been shown that deletion of *HSP60* in adult mice hearts altered mitochondrial complex activity, mitochondrial membrane potential, and reactive oxygen species (ROS) production, and ultimately led to cardiomyopathy and heart failure **(Fan F et al.2020)**.

5.2.5 HSP60 in cancer

HSP60 proteins were highly expressed in tumor cells, such as stomach cancer, colon cancer, liver cancer, breast cancer, and lung cancer. Its abnormal expression is associated with tumour cell metastasis and drug resistance. Recently, cancer researches focused on HSP60 due to its potential as a promising biomarker for diagnosis and a potentially useful target for treatment (**Sun B et al. 2021** and **Michaluart P et al. 2008**).

5.3 HSP 70

The 70-kDa heat shock protein (*HSP70*) family of molecular chaperones represents one of the most ubiquitous classes of chaperones. They are monomeric proteins that reside in any adenosine-5′-triphosphate (ATP)-containing eukaryotic intracellular compartment and can also be found in cell membranes and the extracellular milieu as well as in bacteria **(Radons J et al.2016)**.

5.3.1 Genes

The human *HSP70* family comprises 13 gene products that differ from each other by expression level, subcellular location, and amino acid constitution. The Functional genes encoding human *HSP70* proteins are: *HSPA1A, HSPA1B, HSPA1L, HSPA2, HSPA5, HSPA6, HSPA7, HSPA8, HSPA9, HSPA12A, HSPA 12B, HSPA13, HSPA14* (**Brocchieri et al. 2008**).

5.3.2 Proteins

Humans and eukaryotic organisms express several slightly different HSP70 proteins, the most important of which are: HSP70, HSC70, GRP75, and GRP78. HSP70 family members have three major functional domains: an N-terminal ATPase-binding domain (ABD) responsible for substrate binding and refolding and a C-terminal substrate-binding domain (SBD) to facilitate the release of client protein after ATP hydrolysis. HSC70 (encoded by *HSPA8*) exhibits essential housekeeping functions such as folding and transport of polypeptides across intracellular membranes. HSP70 (encoded by *HSPA1A* and *HSPA1B*) is produced largely by cells in response to hyperthermia, oxidative stress, and changes in pH. GRP75 (encoded by *HSPA9*) regulates the transfer of Ca^{2+} from ER stores into the mitochondrial matrix. GRP78 (encoded by *HSPA5*) facilitates the transport and folding of nascent polypeptides into the ER lumen, and can be upregulated in response to stress or starvation.

HSP70 proteins regulate the activity of multiple kinases, activate and inhibit multiple signaling pathways. They exhibit regulatory functions to c-Jun N-terminal kinases and ERK kinases, as well as regulation of the p38MAPK signaling pathway (**Lee J et al. 2005** and **Fan W et al. 2018**)

5.3.3 Expression

Some members of HSP70 family are constitutively expressed in all human cell types like GRP75 and GRP78. However, HSP70 protein is highly expressed in testis, in brain, kidney, heart, liver and muscle (**Radons J et al. 2016)**. HSP70 members can be found in the mitochondria-associated membrane (GRP75), in the endoplasmic reticulum (GRP78) and in the cytosol (HSP70).

5.3.4 Physiological roles of HSP70 and its genetic deletion

HSP70s were found to have an important role in spermatogenesis and sperm function (**Payan-Carreira R et al. 2020**). In addition, evidence suggests HSP70 to have a range of functions in muscle biology. HSP70 expression is induced in skeletal muscle in response to a variety of physiological injuries, and plays a significant role in protecting cells from damage and dysfunction. *HSP70* genetic deletion alters skeletal muscle fiber size and quality and impairs muscle regeneration and recovery following injury. In addition, *HSP70* deletion might induce cardiac dysfunction and development of cardiac hypertrophy (**Kim YK et al. 2006**).

5.3.5 HSP70 in cancer

HSP70 plays a pivotal role in carcinogenesis acting as a potential tumor biomarker. In cancer cells, HSP70 is constitutively overexpressed and participates in cancer cell survival, tumorigenicity, and anti-apoptotic activities. Furthermore, it has been shown that high expression of this chaperone correlates with increased tumor grade and poor prognosis. HSP70 is overexpressed in patients with non-small cell lung carcinoma (NSCLC) (**Malusecka E et al. 2001**), breast, endometrial, and uterine cervical carcinoma (**Ciocca DR and Calderwood SK. 2005**), as well as in patients with acute myeloid leukemia (**Thomas X et al. 2005**), colorectal carcinoma, prostate and hepatocellular carcinoma (**Abe M et al. 2004** and **Chuma M et al. 2003**). Due to the important role of HSP70 in cancer, several groups focused on the discovery of HSP70 inhibitors for cancer therapy.

5.4 HSP110

Heat shock protein 110 *(*HSP110), also called HSP105 or *HSPH1*, is a ubiquitous chaperone with anti-aggregation capabilities. It is a nucleotide exchange factor for HSP70, this latter relies on it to complete the protein folding and contributing to protein homeostasis (**Mattoo R et al. 2013**). In addition, HSP110 enhances certain signaling pathways like the proliferative Wnt/β-Catenin pathway (**Yu N et al. 2015**) and it activates certain transcription factors like STAT3 (**Berthenet K et al. 2017**). Both the protein homeostasis function and the role of HSP110 on proliferative pathways may explain how this protein is linked to aggressive tumors. Also a strong direct association has also emerged between the nuclear localization of HSP110 and active tumor cells (**Causse S et al. 2018**).

5.4.1 Genes

HSP110 in humans is encoded by the *HSPH1* gene

5.4.2 Proteins

HSP110/105 belongs to the HSP110 heat shock protein family, which is known to have diverged from the HSP70 family and consists of 3 members: HSP110/105, Apg-1 (ATP and peptide-binding protein in germ cells-1) and Apg-2.

5.4.3 Expression

HSP110 is constitutively expressed in mammals in various tissues such as liver, ovary, spleen, heart, lung, small intestine, and muscles, but it shows particularly significant levels in all cerebral neuronal regions, including those in the cerebral cortex and the hippocampus (brain) (**Zuo D et al. 2016)**. HSP110 members are expressed in the cytoplasm and in the nucleus of cells.

5.4.4 Physiological roles of HSP110

Little is known about the roles of HSP110. Beside its role as chaperone, HSP110 has been shown to regulate biogenesis and quality control of misfolded cystic fibrosis transmembrane conductance regulator.

5.4.5 HSP110 in cancer

HSP110 is highly expressed in gastric cancer, colorectal cancer and in B-cell lymphoma **(Kimura A et al. 2016** and **Jego G et al. 2019)**. HSP110 has been shown to be involved in multiple neoplastic processes, and its high expression is associated with cancer progression and poor prognosis. New studies focus on HSP110 as a promising tumor regression agent and a potentially beneficial target for therapy (**Gozzi G et al. 2020**).

5.5 HSP90

The heat shock protein 90 (HSP90) family of proteins is a cluster of highly conserved ubiquitous molecules with an approximate molecular weight of 90-kDa, that are involved in myriad cellular processes (**Table 7**).

5.5.1 Genes

HSP90 family includes 17genes that fall into four classes: *HSP90AA, HSP90AB, HSP90B and TRAP. Six genes, HSP90AA1, HSP90AA2, HSP90N, HSP90AB1, HSP90B1 and TRAP1*, were recognized as functional, and the remaining 11 genes were considered pseudogenes.

5.5.2 Proteins

Mammalian cells express several HSP90 proteins, which are: HSP90α, HSP90β, GRP94, and TRAP1. In mammals, cell proliferation and differentiation are regulated by both HSP90α and HSP90β, mainly by HSP90β, the most important member of the HSP90 family **(Sreedhar A et al.**

2004 and **Voss A.K. et al. 2000)**. HSP90 proteins have wide range of client proteins that are involved in numerous cellular pathways. The cytoplasmic HSP90 (HSP90α and HSP90β) interact with more clients than GRP94 and TRAP1. These clients includes kinases such as Akt2, CDKs, PKC, many MAP kinases as well as transcription factors like steroid receptors, BCL-6, CAR, p53, Oct4 (**Hoter A et al. 2018)**.

5.5.3 Expression

HSP90 are expressed at low levels in muscle; however they are expressed at highest levels in neural regions, in testes, thymus and other tissues such as liver, kidney, heart, and small intestine. The constitutively expressed HSP90β (*HSP90AB1*) and the heat-inducible *HSP90α* (*HSP90AA1*) are mainly localized in the cytoplasm, while GRP94 *(*Endoplasmin) and *TRAP1* (**T**NF **R**eceptor-**A**ssociated **P**rotein1) reside in the endoplasmic reticulum and the mitochondria, respectively (**Chen B et al. 2005)**

Family	Proteins members	Gene encoding	Subcellular location	Function
HSP90A	HSP90α1	HSP90AA1	*Cytosol*	growth promotion cell cycle regulation cellular transformation signal transduction long-term cell adaptation
	HSP90α2	HSP90AA2	*Cytosol*	
	HSP90β	HSP90AB1	*Cytosol*	
HSP90B	GRP94	HSP90B1	*Endoplasmic reticulum*	Immunity regulation
TRAP	TRAP1	TRAP1	*Mitochondria*	Cell cycle regulation

Table 7: Summary of *HSP90* family members and their cellular location and functions.

5.5.4 Structure of HSP90

HSP90 protein is composed of 4 identifiable domains (**Pearl L and Prodromou C. 2006**) (**Figure 13**):

- The N-Terminal Domain—Nucleotide and Drug Binding: this structure consists of residues 1-220 (yeast), it is very highly conserved in sequence among the *HSP90* family. This domain binds adenine nucleotides and is essential for the ATP-dependent function of the chaperone in vivo. In the human structure, this pocket was found to be the binding site for the *HSP90* inhibitors like geldanamycin (antitumor agent), whose binding to *HSP90* had been shown to disrupt productive complexes with protein kinase and steroid hormone receptor clients.
- "Charged linker" region: a highly charged and proteolytically sensitive segment that connect the N-terminal nucleotide-binding domain to the remainder of the protein.
- The middle Segment—Client Protein Binding and Catalytic Loop(38-44 kDa): this structure consists of a large αβα domain at the N terminus of the construct connecting to a small αβα domain at the C terminus via a series of short α helices in a tight coil. It is also involved in client protein binding.
- C-terminal domain —Dimerization (~12kDa): provides a strong inherent dimerization interface, which is essential for function. It provides the binding site for a subset of HSP90 co-chaperones. Although it is involvement in function, however its removal has no significant effect on the inherent ATPase activity of HSP90 in vitro.

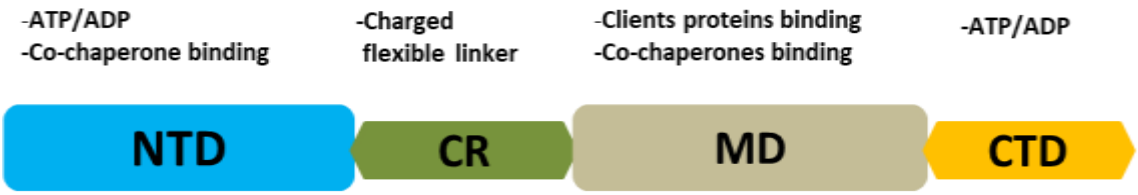

Figure 13: Domain structure of HSP90 family members. Schematic representation of the domain structure of HSP90 and the function of each domain .Drawn according to information from (HU L et al.2020)

5.5.5 Mechanism

- ATP binding : the region of the protein near the N-terminus has a high-affinity ATP-binding site. The ATP binds to a sizable cleft in the side of protein. This cleft has a high affinity for ATP, and when given a suitable protein substrate, *HSP90* cleaves the ATP into ADP and P_i. Direct inhibitors of ATP binding or allosteric inhibitors of either ATP binding or ATPase activity can block *HSP90* function (**Goetz M et al. 2003**).
- Protein binding : the protein-binding region of HSP90 is located toward the C-terminus of the amino sequence. The *HSP90* protein can adopt two major conformational states. The first is

an open ATP-bound state and the second is a closed ADP-bound state. Thus, ATP hydrolysis drives what is commonly referred to as a “pincer-type” conformational change in the protein binding site.

HSP90 is responsible for the maturation and activation of its client proteins including regulatory kinases, steroid hormone receptors and transcription factors, through an ATPase-driven cycle. It also plays a role in the degradation of irreparable proteins through involvement in the ubiquitin–proteasome pathway mediated by the carboxyl terminus of HSP70-interacting protein (**Pratt W et al. 2010**).

The HSP90 cycle is controlled by co-chaperones like HOP, AHA1, CDC37 and P23 in various ways, such as the inhibition and activation of its ATPase activity in addition to recruitment of specific client proteins. A simplified chaperone cycle of HSP90 is represented in **figure 14 (Li J et al. 2012)**

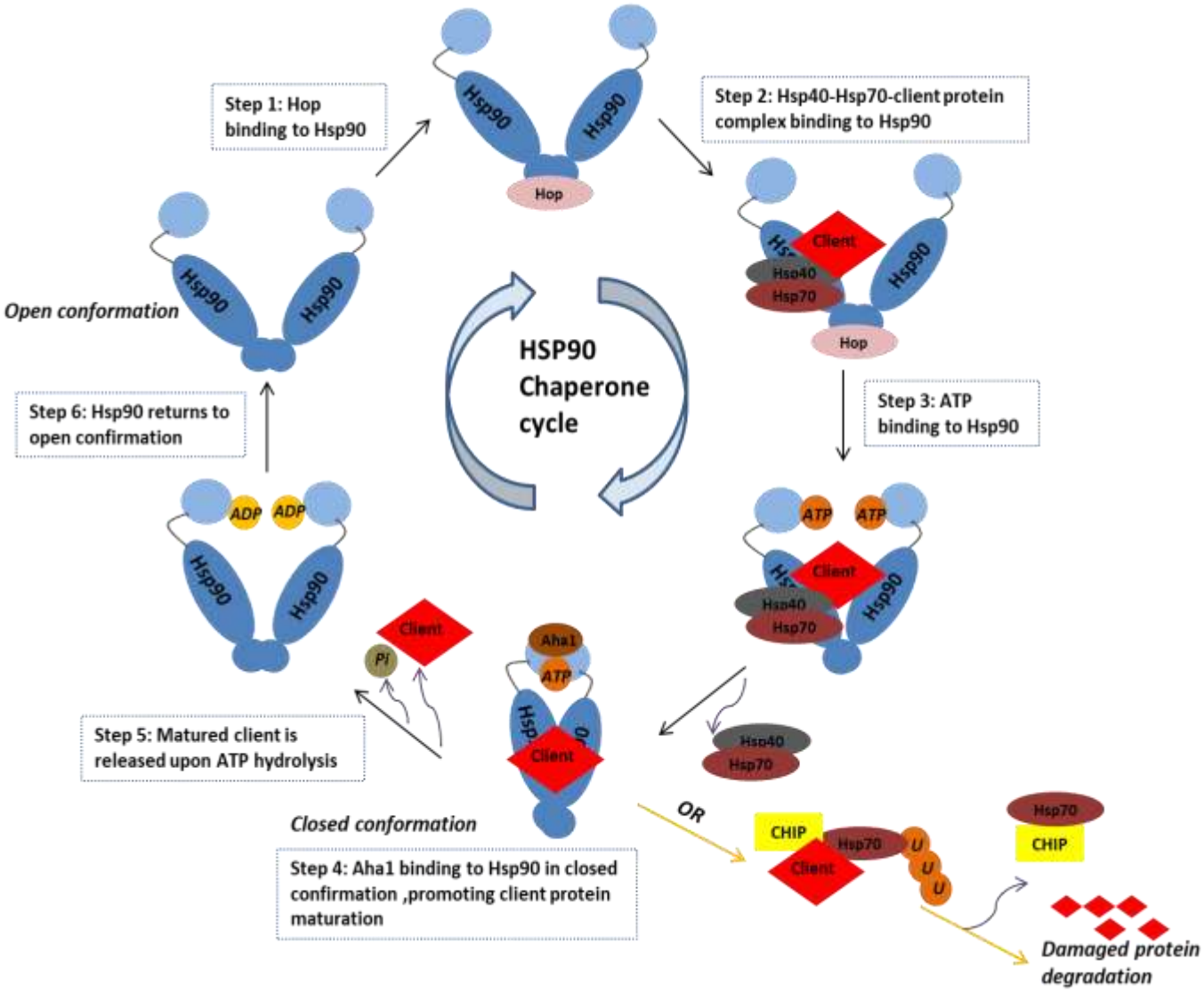

Figure 14: The Chaperone Cycle of HSP90. HSP90 functions in concert with a cohort of chaperones and co-chaperones to fold, activate and mature its client proteins. The chaperone cycle begins with the binding of co-chaperone Hop to HSP90 in the 'open' conformation. Hop would then recruit the HSP40-HSP70-client protein complex and assist in loading client protein onto HSP90. Subsequent binding of ATP induces dimerization at the N-terminal domain of HSP90 resulting in the 'closed' conformation. The co-chaperone Activator of HSP90 ATPase 1 (Aha1) binds to the N-terminal domain of HSP90 and stabilizes the 'closed' conformation promoting the maturation of client protein. Upon the hydrolysis of ATP, HSP90 returns back to the 'open' conformation and the matured client protein is released. HSP90 also degrades irreparable proteins through involvement in the ubiquitin–proteasome pathway .Drawn with information from (Hu Li et al. 2020).

5.5.6 HSP 90 and Cancer

In normal cells, HSP90α and HSP90β are present in the cytoplasm where they interact with client proteins (**Sreedhar A et al. 2003** and **Hoter A et al. 2018**). Their levels are lower in normal cells than in neoplastic cells (**Sreedhar A et al. 2004**).

Heat-shock protein 90 and other chaperones are found expressed two to ten times greater in many solid tumors and hematological malignancies than in normal cells (**Solárová Z et al. 2014)**. HSP90 expression may in part account for the ability of cancer cells to maintain protein homeostasis, by stabilizing mutated and overexpressed oncoproteins, such as BCR-ABL, EGFR, ERK, FLT3, HER2, CDK4, CDK6, MEK, RAF, JAK2, STAT3 and SRC even in the hostile hypoxic and acidotic microenvironment of the tumor (**Whitesell L and Lindquist S. 2005**). **Figure 15** shows the relationship between HSP90 and these client proteins.

HSP90 α and β are highly expressed in breast cancer, hepatocellular and pancreatic carcinoma, lung cancer and leukemia (**Meng J et al. 2017**; **Rong B and Yan S. 2018**). HSP90 expression has also been shown to be associated with chronic tumors (**Rong B and Yan S. 2018).**
In addition to the fact that HSP90 α and β are essential for proper functioning, facilitating the growth and survival of cancer cells, moreover, they also have a specific role in maintenance of cancer cell by inhibiting apoptosis and this explains the aggressiveness and the resistance of cancer cells to chemotherapy and radiation (particularly HSP90 β) (**Sreedhar A et al. 2004** and **Tanno S et al. 2004**). The importance of HSP90 in cancer progression has been demonstrated in several studies, showing that deletion or inhibition of HSP90 by HSP90 inhibitors reduces cancer cell development and metastasis and induces apoptosis of these cells (**Mori M et al. 2015**). Recently, the inhibition of HSP90 in HER2-positive breast cancer cell lines has been shown to prevent tumor growth and angiogenesis and induce apoptosis, by decreasing the expression of HER2 (**Park J et al. 2020**).

5.5.7 HSP90 and Leukemia

Several studies have reported that HSP90 levels are elevated in CML and AML cells (**Khajapeer K et al. 2015** and **Sanil B et al. 2018**), and that these elevated levels of HSP90 could serve as prognostic marker in these types of leukemia (**Zackova M et al. 2013**). Trentin L et al, found that

HSP90, is overexpressed in B-CLL compared with resting B cells (**Trentin L et al. 2008** and **Guo A et al. 2017**). Other studies reported that the expression of HSP90 in patients with ALL was significantly higher than that in controls and the presence of a strong HSP90 expression was associated with a low survival rate (**Hacıhanefioglu A et al. 2011**). Furthermore, studies reported that plasma HSP90 was validated as a soluble biomarker of ALL, useful for earlier detection of leukemia engraftment (**Milani M et al.2015** and **Pawlik-Gwozdecka D et al. 2020**).

It has been shown that human UKE-1 leukemia cells expressing an active mutation of JAK2, exhibited a higher sensitivity to HSP90 inhibitor (PU-H71),and that treatment with HSP90 inhibitor was associated with induction of apoptosis and inhibition of proliferation of leukemic cells by the dephosphorylation of JAK2 and its related downstream pathways **(Ho N et al. 2012).** Another study showed that the inhibition of HSP90 by 17AAG (HSP90 inhibitor), induced apoptosis in AML blasts expressing a high level of FLT3, via degradation of the latter (**George P et al. 2004**). Furthermore, Chatterjee M et al showed that knockdown of *HSP90* by shRNA and its inhibition by HSP90 inhibitors alter the level of STAT3 and phospho-ERK and induce the apoptosis of multiple myeloma cells (**Chatterjee M et al. 2007**).

In addition to studies mentioned above, it was confirmed that the inhibition of HSP90 leads to inhibition and proteasomal degradation of many deregulated oncoproteins that are believed to be critical for all fundamental hallmarks of cancer including proliferation, evasion of apoptosis, immortalization, invasion, angiogenesis and metastasis (**Neckers L and Workman P. 2012**). On the other side, these observations led us to hypothesize that LCK and LYN, which are included in leukemia and tumor formation could rely on HSP90 to chaperon and to maintain the oncogenic pathways.

5.5.8 HSP90 and SRC kinases

HSP90 has a special role among “signaling-chaperones”; it is a key organizer of several cytoplasmic complexes. HSP90 binds to steroid receptors and to several serine and tyrosine kinases including the SRC, RAF, focal adhesion kinases and protein kinase CK-II, or cyclin dependent kinases-4, 6 and 9 (**Baker J et al. 2019** and **Hoter A et al. 2018**), which are central players in key signal transduction pathways. Since we are working on T-ALL, we have been interested in T lymphocytes.

Studies have shown that activation of T lymphocytes results in the tyrosine phosphorylation of numerous CD3 immunoreceptor tyrosine-based activation motifs and a consequent recruitment and phosphorylation of downstream substrates, adaptor proteins, and protein kinases (**Schnaider T et al. 2000**). During this process, the SRC family protein tyrosine kinase LCK was found to be critical for T-cell receptor (TCR) signaling. The heat shock protein (HSP90) and its cochaperones were found necessary to achieve the signal-competent conformation of LCK and other elements of T-cell signaling (**Schnaider T et al. 2000**). The involvement of HSP90 in the synthesis, membrane binding, and maintenance of the SRC-kinase LCK was studied using specific inhibitors of HSP90, like Geldanamycin and Radicicol. They found that functional HSP90 is essential for the stability and function of LCK, similar results were obtained for other SRC tyrosine kinases like c-SRC (**Bijlmakers MJ and Marsh M. 2000**; **Giannini A and Bijlmakers MJ. 2004**).

Since we work also on B-ALL, we have been interested in B lymphocytes. Antigen (Ag) binding to surface immunoglobulins (sIg) triggers BCR (B-Cell Receptor) activation by inducing its translocation to lipid rafts, where the co-stimulating immunoglobulins heterodimer (CD79a/b) is phosphorylated by LYN kinase. Guo A et al demonstrated an association between heat shock protein 90 and SRC tyrosine kinase LYN, in a multi-client chaperone complex. Integrity of this HSP90-LYN complex appears to be critical for maintenance of tonic BCR signaling (**Guo A et al. 2018**).

Several studies showed that HSP90 controls function of LCK and LYN ,which are overexpressed in T-cell and B-cell acute lymphoblastic leukemia, consequently, we decided to test the efficacy of NVP-BEP800, a novel HSP90 inhibitor in inhibiting LCK and LYN, and in reducing the proliferation of leukemic cells in T-ALL and B-ALL.

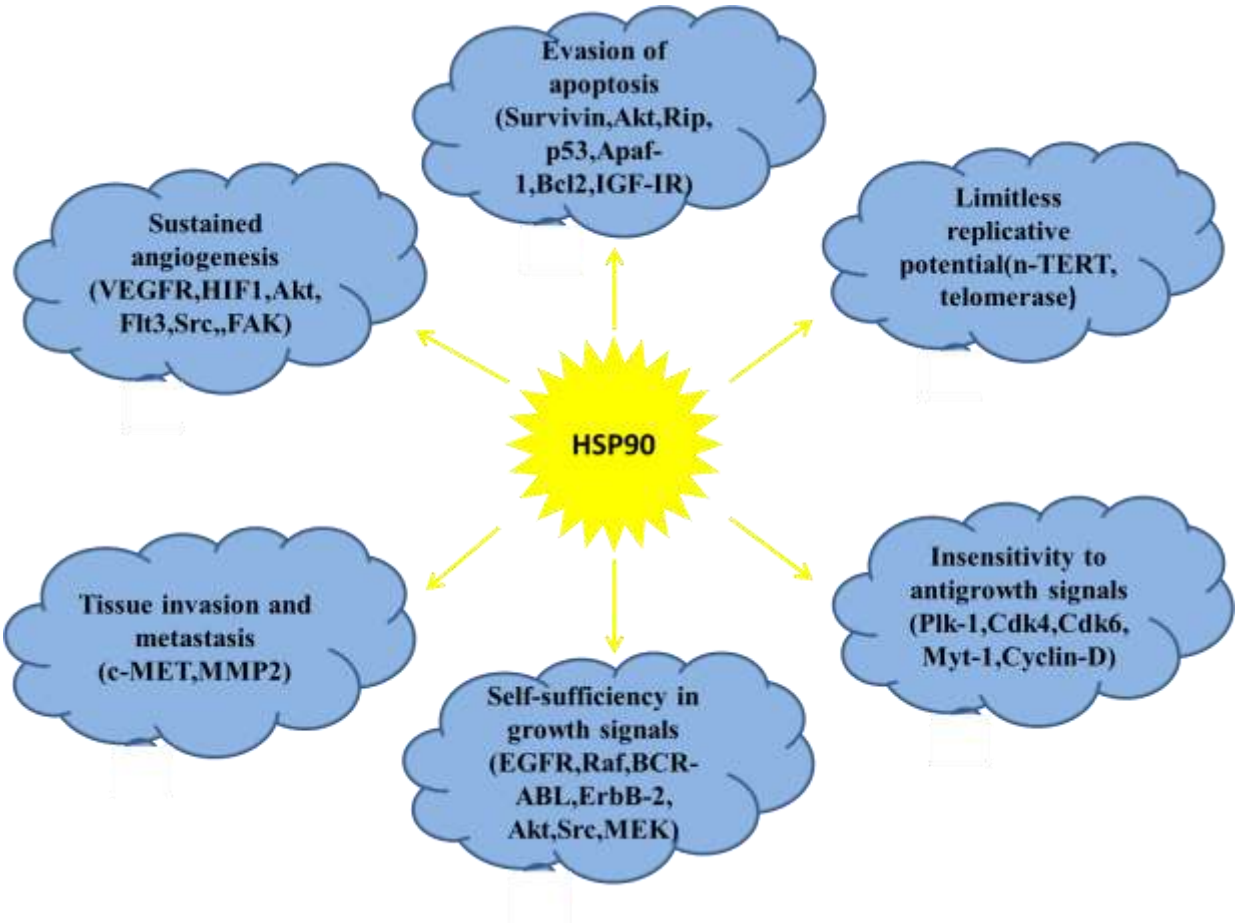

Figure 15: Role of HSP90 and its clients in cancer progression and metastasis. Relationship between HSP90 and various client proteins resulting in cancer cell survival, progression, invasion, and metastasis. Drawn with information from (Khajapeer KV and Baskaran R et al. 2015).

6 HSP 90 inhibitors - potential therapeutic agents

First, *HSP90* is known to facilitate the maturation, stabilization and activation of over 200 client proteins, covering all cellular processes (**Table 8**) (**Taipale M et al. 2010** and **Normant E et al. 2011**).

Huge efforts have been made over the past several decades to develop selective inhibitors that directly target HSP90 or HSP90 cancer-related complexes. Structurally, HSP90 inhibitors can be roughly categorized into three chemotypes, including (1) HSP90 N-terminal inhibitors (ATP Competitive inhibitors), (2) C-terminal HSP90 inhibitors, and (3) competitive HSP90 inhibitors other than ATP. However, the majority of HSP90 inhibitors that are currently available, and all that have

been clinically assessed, bind to the nucleotide binding pocket of the N-terminal domain and block the processing of client proteins by preventing ATP binding and hydrolysis (**Garg G et al. 2016**).

The first HSP90 inhibitors identified was the bacterial-derived benzoquinone ansamycin Geldanamycin (GA). Geldanamycin mimics ATP and binds to the nucleotide-binding pocket on the N-terminus of HSP90, blocking the natural substrate ATP binding and destabilizing the client protein like “SRC” (**Miyata Y. 2005**). Although GA exhibits very strongly antitumor activities in vitro, it was never evaluated in the clinic because of its poor solubility in aqueous solutions and its toxicity (**Messaoudi S et al. 2011**). Therefore, GA derivatives with comparable antitumor effects and even better toxicological properties have entered into the clinical trials (at the beginning of 2014), and of these derivatives we have: 17-AAG (Tanespimycin) that entered to the third phase of the clinical trials, 17-DMAG (Alvespimycin), IPI-504 (Retaspimycin hydrochloride) and most recently 17-amino-17-demethoxyGeldanamycin (17-AG).

Another intensively studied N-terminal domain binder of HSP90 is Radicicol, a macrocyclic natural antibiotic isolated from the fungus Monocillium nordinii and from the plant-associated fungus Chaetomium chiversii (**Turbyville T et al. 2006**). Newly created stable oxime derivatives of Radicicol and cykloproparadicicol have shown greater potency and favorable toxicity profile (**Soga S et al. 2003**). Moreover, resorcinol-based synthetic agents like NVP-AUY922 (luminespib; Vernalis, formerly Novartis) is currently in Phase II, STA-9090 (Ganetespib, Syntha Pharmaceuticals) is currently in Phase III clinical development (**Butler L et al. 2015**). Additional agents are undergoing preclinical development and predicted to enter the clinic in the near future. Among these agents, a novel series of orally bioavailable 2-aminothieno [2,3-d]pyrimidine–based HSP90 inhibitor was discovered(NVP-BEP800). In my thesis I tried to explore the potency and efficacy of this newly discovered HSP90 inhibitor (NVP-BEP800) in treating B-ALL and T-ALL patients.

Transcription factors	Kinases	Others
AF9/MLLT3 , BBX ,BCL-6	***Akt/PKB, BCR-ABL ,BLK , BTK***	***Apaf-1 ,apoB ,APOBEC-3B, 3C, 3G ,ARD1 , Argonaute-1 (Ago1)***
Steroid receptors (GR, MR, ERa, ERb, PR, AR)	***CDK1,CDK2, CDK4, CDK6, CDK9,***	***Bcl-2 , Bcl-xL, calcineurin ,calmodulin***
HSF-1, HsfA1, HsfA2, HsfB	***EGF receptor (mutant and wt) , eIF2-a kinases HRI, Gcn2,***	***Fibronectin - FliN, Folliculin , Gln1 - GLT-1***
c-Myc, NF-KB	***Flt3,FYN,HCK ,JNK***	***MMP2, MMP3, MMP9***
Notch1 ,NR1H3 , NR1I2	***LCK, LYN and other SRC related tyrosine kinases: fer, fes, fgr, fps, , yes***	***Nox1, Nox2, Nox3, Nox5***
p53 - p73	***mTOR , MAP2K5 ,MAP2K7, MAP3K12 , MAP3K15***	***Survivin , SV40 large T-antigen , Swr1 , a-synuclein ,TCL1A , telomerase***
Stat2, Stat3, Stat5 and others.	***Pim-1 , PIM2 ,PIM3 ,SYK ,ZAP-70 and others***	***VIP1 , VPS18, ZMYND10***

Table 8: Different HSP90 client proteins

6.1 NVP-BEP800

NVP-BEP800 represents a novel fully synthetic, orally available 2-aminothieno [2,3-d] pyrimidine class inhibitor that binds to the NH2-terminal ATP-binding pocket of HSP90 (**Figure 16**) (**Brough P et al. 2009; Stühmer T et al. 2009; Massey A et al. 2010**). It is a potent inhibitor of HSP90β (*HSP90AB1*) and is 70 times less effective against the related *HSPs*: GRP94 *(HSP90B1*) and TRAP1 *(HSP90L)*. The compound has favorable pharmaceutical and pharmacological properties. It is reported to demonstrate strong antiproliferative activity against various tumor cell lines at tolerable doses and primary tumors in vitro and in vivo (**Massey A et al. 2010**). NVP-BEP800 causes p23/HSP90 dissociation, client protein degradation as well as the reduction of client protein phosphorylation, which leads to growth inhibition and induction of cell death in cancer cells.

Additionally, it radiosensitises tumor cells through cell-cycle impairment (**Stingl L et al. 2010**); and directs the proteasomal degradation of viral HSP90 client proteins, including those required for latency and infectivity of Kaposi sarcoma-associated herpes virus.

Beside its strong antiproliferative activity against tumor cells, NVP-BEP800 has been shown to inhibit the proliferation of malignant glioblastoma cells (**Wu J et al. 2014**) and to induce apoptosis of myeloma cells (**Stühmer T et al. 2009**). However, to date, no study has been performed to test the efficacy of NVP-BEP800 in treating acute lymphoblastic leukemia.

My present work tested the effect of the novel 2-aminothienopyrimidine class HSP90 inhibitor, NVP-BEP800, on the survival of B-ALL and T-ALL cells in vitro and in vivo as preclinical studies.

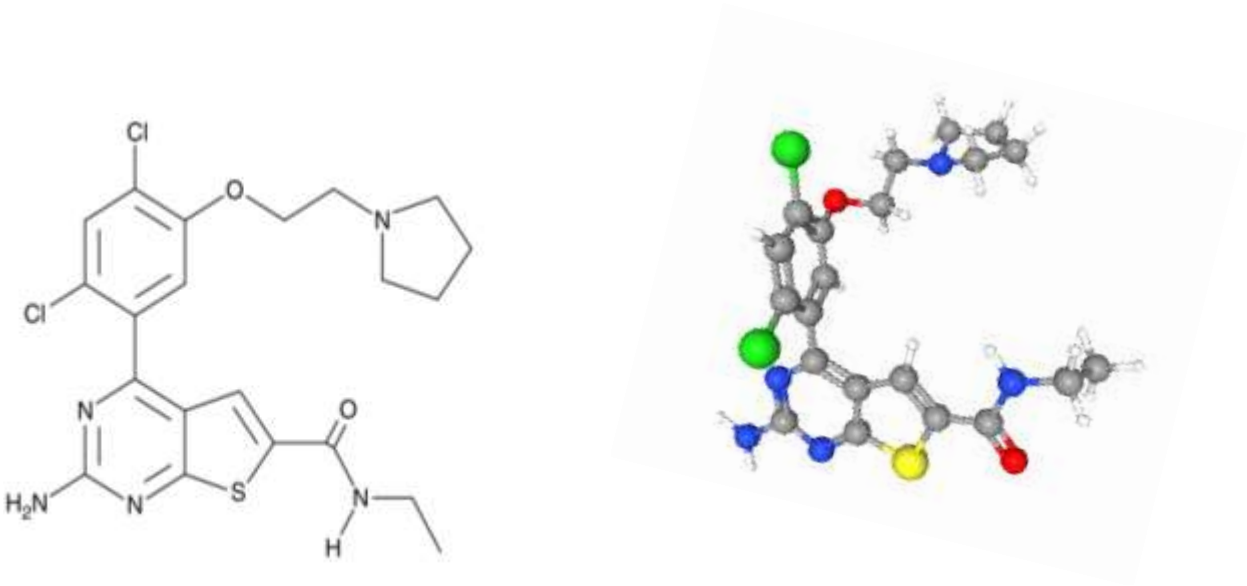

Figure 16: Chemical structures of NVP-BEP800 (2-aminothienopyrimidine)

Objectives of thesis:

Our main hypothesis is that inhibition of HSP90, the chaperone protein of SRC kinases, by HSP90 inhibitors could be a more effective approach in treating acute lymphoblastic than the use of SRC inhibitors.

My thesis has many goals that I will try to achieve.

First, I will check whether HSP90 and SRC kinases are highly expressed B-ALL and T-ALL cells. Second, I will try to demonstrate that HSP90 is a chaperone of LCK and LYN and then I will test whether HSP90 inhibitors can inhibit the survival and proliferation of T-ALL and B-ALL cells in vitro, on several lymphoid cell lines. Next, I will identify the SRC signaling pathways involved in leukemia development and the effect of NVP BEP800 on these pathways. Later, I will study the correlation between the presence of LCK and LYN in leukemic cells and their sensitivities to NVP-BEP800. Finally, I will evaluate the anti-leukemic effect of NVP-BEP800 in vivo, on several xenografted mice models.

II.RESULTS

I. HSP90 inhibitor NVP-BEP800 affects stability of SRC kinases and growth of T-cell and B-cell acute lymphoblastic leukemias

This project is part of the objective of discovering innovative therapeutic avenues to treat T-ALL and B-ALL. Thanks to a collaboration with people who worked on acute lymphoblastic leukemia, we were able to build T-ALL and B-ALL xenograft models by injecting NSG mice with primary T-ALL and B-ALL cells obtained from the Biological Resources Center of the CHU of Dijon.

By proteomic analysis and flow cytometry, we were able to demonstrate that bone marrow cells from PDX models of T-ALL and B-ALL express a high level of p-LCK and p-LYN; also both models highly express HSP90. Then by fluorescence microscopy and immunoprecipitation we showed that LCK binds to HSP90 in PDX T-ALL cells and LYN binds to HSP90 in PDX B-ALL cells.

Due to these findings we treated ALL cells with NVP-BEP800, an inhibitor of HSP90, and we found that NVP-BEP800 affected the ALL cells in vitro and in vivo by affecting the stability of SRC kinases involved in signaling pathways necessary for survival, growth and maintenance of ALL cells.

These results, presented in a publication published in Blood Cancer Journal in March 2021, make it possible to consider a new therapeutic strategy to treat ALL.

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HSP90 inhibitor NVP-BEP800 affects stability of SRC kinases and growth of T-cell and B-cell acute lymphoblastic leukemias

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Abstract

T-cell and B-cell acute lymphoblastic leukemias (T-ALL, B-ALL) are aggressive hematological malignancies characterized by an accumulation of immature T- or B-cells. Although patient outcomes have improved, novel targeted therapies are needed to reduce the intensity of chemotherapy and improve the prognosis of high-risk patients. Using cell lines, primary cells and patient-derived xenograft (PDX) models, we demonstrate that ALL cells viability is sensitive to NVP-BEP800, an ATP-competitive inhibitor of Heat shock protein 90 (HSP90). Furthermore, we reveal that lymphocyte-specific SRC family kinases (SFK) are important clients of the HSP90 chaperone in ALL. When PDX mice are treated with NVP-BEP800, we found that there is a decrease in ALL progression. Together, these results demonstrate that the chaperoning of SFK by HSP90 is involved in the growth of ALL. These novel findings provide an alternative approach to target SRC kinases and could be used for the development of new treatment strategies for ALL.

Introduction

Acute lymphoblastic leukemia (ALL) is a type of cancer that leads to the proliferation of immature hematopoietic cells due to genetic alterations in lymphocyte precursors[1–6]. ALL is a heterogeneous disease that affects T- or B-lymphocyte precursors in 25 and 75% of cases, respectively. ALL accounts for approximately 12% of all cases of leukemia, which represents about one or two new events per 100,000 inhabitants per year. ALL mostly affects children aged two to five years, with 75% of patients under the age of eighteen. It accounts for approximately 30% of childhood cancers and for 80% of all leukemia cases in children. ALL also affects adults but to a lesser extent[7,8]. Thanks to improvements in the available treatments for ALL[1–6], complete remission rates have become high, exceeding 80% in children and 50% in adults. However, long-term survival at ten years (event-free survival) is in the range of 60–80% for children and 25–35% for adults. This implies that there is still a significant need for new therapies to maintain remission and prolong survival. It is therefore necessary to improve our knowledge of this condition in order to discover new therapeutic strategies that would reduce the intensity of cytotoxic chemotherapy and improve the prognosis of patients after a relapse.

The chaperone Heat shock protein 90 (HSP90) plays a role in protecting the proper three-dimensional folding of proteins. HSP90 was found overexpressed in leukemia cells[9], and its high expression was necessary for the survival and propagation of cancer cells. Treatments using HSP90 inhibitors that have been developed for solid tumors[10–13] have therefore also been used for hematological disorders[14]. HSP90 inhibition has, for instance, been shown to be efficient for the treatment of lymphomas[15–17].

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Several HSP90 inhibitors can overcome the resistance to Fms-like tyrosine kinase 3 (FLT3) inhibitors that has been observed in acute myeloid leukemia (AML)[18]. For the treatment of AML, the HSP90 inhibitor NVP-AUY922 has shown synergistic anti-leukemic activity with Cytarabine in vivo[19], and Ganetespib (STA-9090) has also been tested in combination with Cytarabine as a potential active agent[20]. Alvespimycin (17-DMAG) administered intravenously twice weekly to AML patients was also found to be effective[21]. Co-treatments with 17-Allylamino-17-demethoxygeldanamycin (17-AAG) and FLT3 kinase or Histone deacetylase inhibitors were highly effective against human AML cells with mutant FLT3[22,23]. Elevated HSP90 inhibition disrupted JAK-STAT signaling and led to a reduction in splenomegaly in patients with myeloproliferative neoplasms[24]. HSP90 inhibitor was synergistic with JAK2 inhibitor and overcame resistance in human myeloproliferative neoplasm cells[25]. Other studies have also confirmed that there was an elevated expression of HSP90 in chronic myeloblastic leukemia (CML), suggesting that HSP90 could serve as a prognostic marker[26]. This also explains why several chemical inhibitors of HSP90 have been tested to treat CML[27]. In addition, targeting HSP90 dimerization was found effective in imatinib-resistant CML[28].

Regarding ALL, the HSP90 inhibitor PU-H71 has been shown to be effective in treating T-ALL patients samples that express a high level of NOTCH1 (Notch receptor 1)[29], NVP-AUY922 led to a degradation of Tyrosine kinase 2 (TYK2) signaling and T-ALL apoptosis[30]. In a subset of B-ALL, genetic resistance to Janus kinase 2 (JAK2) inhibition was overcome by HSP90 inhibition[31]. HSP90 expression in patients with T-ALL and B-ALL was significantly higher than those in a control group, and strong HSP90 expression was associated with a low survival rate[32]. Furthermore, plasmatic HSP90 has been validated as a soluble biomarker of T-ALL and B-ALL, which can be used for earlier detection of leukemia engraftment and progression in mice[33].

SRC refers to a family of proto-oncogenes encoding the Lymphocyte-specific SRC family kinases (SFK). In this family, LCK (for Lymphocyte-specific protein tyrosine kinase) was highly expressed by T-ALL and was found essential for T-cell receptor (TCR) signaling[34,35]. Glucocorticoid resistance was reversed by LCK inhibition in pediatric T-ALL[36]. The inhibition of LCK, by preventing its phosphorylation, was an important strategy for the treatment of malignant hematopoiesis such as T-ALL, particularly with the use of Bosutinib, Dasatinib, or Saracatinib, which affected the proliferation of leukemia cells[36–38]. Its homolog protein LYN (Lck/Yes-related novel protein tyrosine kinase) was more specifically expressed by B-ALL and was important for B-cell receptor (BCR) signaling[39,40]. The inhibition of LYN was an important strategy for the treatment of B-ALL, more particularly with Dasatinib[41–43]. However, Ibrutinib inhibited BCR positive B-ALL progression by targeting important kinases in the BCR pathway[44]. In leukemia, HSP90 has been shown to bind to LYN in B-chronic lymphoblastic leukemia (B-CLL) and the use of 17-AAG destabilized the binding of HSP90-LYN in vitro, initiating cell apoptosis[45].

Studies have revealed interactions of LCK[46] and LYN[47] with HSP90. The purpose of this project was first to test several HSP90 inhibitors in order to study their ability to deactivate the SFK clients of HSP90 in ALL. While HSP90 inhibitors were often investigated as anti-cancer drugs, we discovered that NVP-BEP800, which acts as an inhibitor of the ATP pocket of HSP90β[48], can inhibit LCK in T-ALL and LYN in B-ALL. Also, we found that this drug reduced the viability of primary T-ALL and B-ALL cells in vitro. In addition, leukemia cell development and proliferation were inhibited in NVP-BEP800 treated xenografted mouse models.

Materials and methods

Patient samples

T-ALL and B-ALL samples, isolated from bone marrow (BM) or peripheral blood (PB) were collected from two independent cohorts in Dijon and Paris. For the first cohort, patients were included at diagnosis or relapse after giving their informed consent (Hôpital Universitaire François Mitterrand, CRB Ferdinand Cabanne, Dijon, France), under the reference number BB-0033-00044, in accordance with the declaration of Helsinki and under clinical trial reference nct04437420. Patients from the second cohort were children or young adults. Samples were included at diagnosis or relapse from the pediatric hematological unit (Dr. Paola Ballerini) at the Assistance Publique Hôpitaux de Paris (APHP, Paris, France), under the reference number CAALL-F01, in accordance with the declaration of Helsinki. Translocations, intrachromosomal deletions, and mutations in T-ALL and B-ALL were identified following specific procedures. The parents or representatives of patients younger than 18 years old gave informed consent.

Establishment of xenograft models

The ethics committee for animal welfare of the University of Burgundy and the French ministry of higher education and research approved all animal experiments (under reference APAFIS#16187-2018071914379464v3). We confirm that all experiments were performed according to the relevant guidelines and regulations of this committee. NOD/SCID/$\gamma c^{-/-}$ (NSG) mice (Charles River) were bred and housed in pathogen-free conditions. Regarding cytogenetic characterization, T-ALL cells transplanted into PDX mice contain a STIL (*SCL/TAL1*

interrupting locus), as well as deletions in *LEF1* and *CDKN2A* genes. Transplanted B-ALL cells, displayed a translocation t(2;8) (p11;q24) *MYC/IGK*. To induce leukemia in mice, we injected 10^5 T-ALL or B-ALL cells in a volume of 300 µl of PBS1×, into the tail vein (intravenous; i.v.) of non-irradiated 7-16-week-old male and female NSG. Mice were treated with NVP-BEP800 (SelleckChem) at 10 mg/kg, with three i.v. injections on the days indicated on the figures. NVP-BEP800 was reconstituted in 100% ethanol at 10 mg/ml and diluted in 300 µl of PBS1× just before it was injected into the mice. Ethanol was the diluent, which served as control "vehicle" in vivo. Mice were randomly allocated to experimental groups and no blinding method was followed for injections. For experiments, we used males and females. There were no animal exclusion criteria. Mice were euthanized when moribund or at the indicated time points. After tail vein PB sampling, hematopoietic cells were counted using a hemocytometer (Vet ABC+, SCIL).

Bioluminescence imaging

We created PDX models that developed stable bioluminescence by infecting T-ALL and B-ALL cells with a lentivirus expressing both GFP and luciferase. Then, we transplanted these cells into mice that were used later to perform bioluminescence imaging. The lentivirus was produced in HEK293 cells after transduction with Lipofectamin 2000 (Thermo Fisher Scientific) of the pCCLc-MNDU3-Luciferase-PGK-EGFP-WPRE vector (Addgene, #89608), as well as PAX2 (Addgene, #12260) and pCMV-VSV-G (Addgene, #8454) plasmids. After two days, viral supernatants were recovered, and six-well plates were incubated 4 h with retronectin (Takara, Ozyme). Viral supernatants were then spinoculated for 30 min at 4,000 g. Cells were cultured on these plates for three days in StemMACS media (Miltenyi Biotech). Lentiviral transduced cells (GFP^+) were sorted on a FACSAriaIII cell sorter (BD Biosciences) and transplanted in NSG mice to generate bioluminescent PDX models. Animals were injected with potassium salt of D-luciferin (150 mg/kg body weight). Following isoflurane-induced anesthesia, animals were imaged 20 min after D-luciferin injection using an IVIS Lumina III system coupled to Living Image acquisition and analysis software version 4.0 (Perkin Elmer).

Statistics

All data were expressed as means ± standard deviation (SD). Differences between two groups were assessed with the two-tailed unpaired Student's *t* test, two-tailed paired Student's *t* test or the Wilcoxon–Mann–Whitney test. The one-way Anova with Tukey's multiple comparison test was used to assess differences between more than two groups. Survival curves were assessed using the Mantel–Haenszel (Log-Rank) test. No statistical methods were used to predetermine the sample size. The variance was similar between the groups that were statistically compared. Statistics were performed using Prism 6 (GraphPad), where significance is indicated on the figures.

Cell culture and treatment with NVP-BEP800, cell viability assay (XTT), western blot, immunoprecipitation, flow cytometry, fluorescent-activated cell sorting (FACS), fluorescence microscopy, immunohistochemistry, quantitative reverse transcription PCR, shRNA lentiviral cloning and viral infection, as well as high-performance liquid chromatography (HPLC) were performed as described in the supplementary materials and methods.

Results

NVP-BEP800 affects viability of lymphoid lines expressing SRC

HSP90 (Heat shock protein 90) is a chaperone protein that modulates intracellular signaling and protein folding. It also stabilizes several other proteins implicated in tumor growth. Lymphocyte-specific SRC family kinases (SFK) are important regulators of pathways involved in the proliferation and growth of lymphoid leukemia cells. Our aim was therefore to test whether HSP90 inhibitors had an effect on the stability of SRC proteins. We focused on inhibitors that target the N-terminal ATP-binding pocket of HSP90 rather than the C-terminal portion, since they were more potent inhibitors[11]. We tested two compounds that target both HSP90α and HSP90β, Luminespib (NVP-AUY922)[49] and 17-AAG[50]. We also tested NVP-BEP800, an inhibitor that was discovered to target only HSP90β[48]. Among the SFK, T-cells expressed more LCK[51], while B-cells expressed more LYN[40]. When we examined the effect of the three compounds on the stability of phosphorylated SRC (active form) and the total amount of SRC proteins, NVP-BEP800 was the most efficient (Fig. 1a). Furthermore, loss of LCK and LYN was observed between 12 and 24 h after the treatment of Jurkat or Raji cells on a time-course experiment (Supplementary Fig. S1). Using the XTT assay to study the viability, we found that ALL cells were more sensitive to NVP-BEP800, than the other two compounds (Fig. 1b). We next used two T-ALL cell lines, the Jurkat line expressing LCK and the Rpmi-8402 line that showed no expression of LCK[51]. Through western blot, NVP-BEP800 was found to affect the stability of phosphorylated LCK and the total amount of LCK in the Jurkat line, while both cell lines were expressing HSP90 (Supplementary Fig. S2a). The XTT assay showed that cells that expressed more LCK (Jurkat) were more sensitive ($P < 0.001$) to NVP-BEP800, compared to non-expressing cells (Rpmi-8402) (Supplementary Fig. S2b). Using four B-lymphoid cell lines (Raji, Daudi, Reh, and BALL-1), western blot

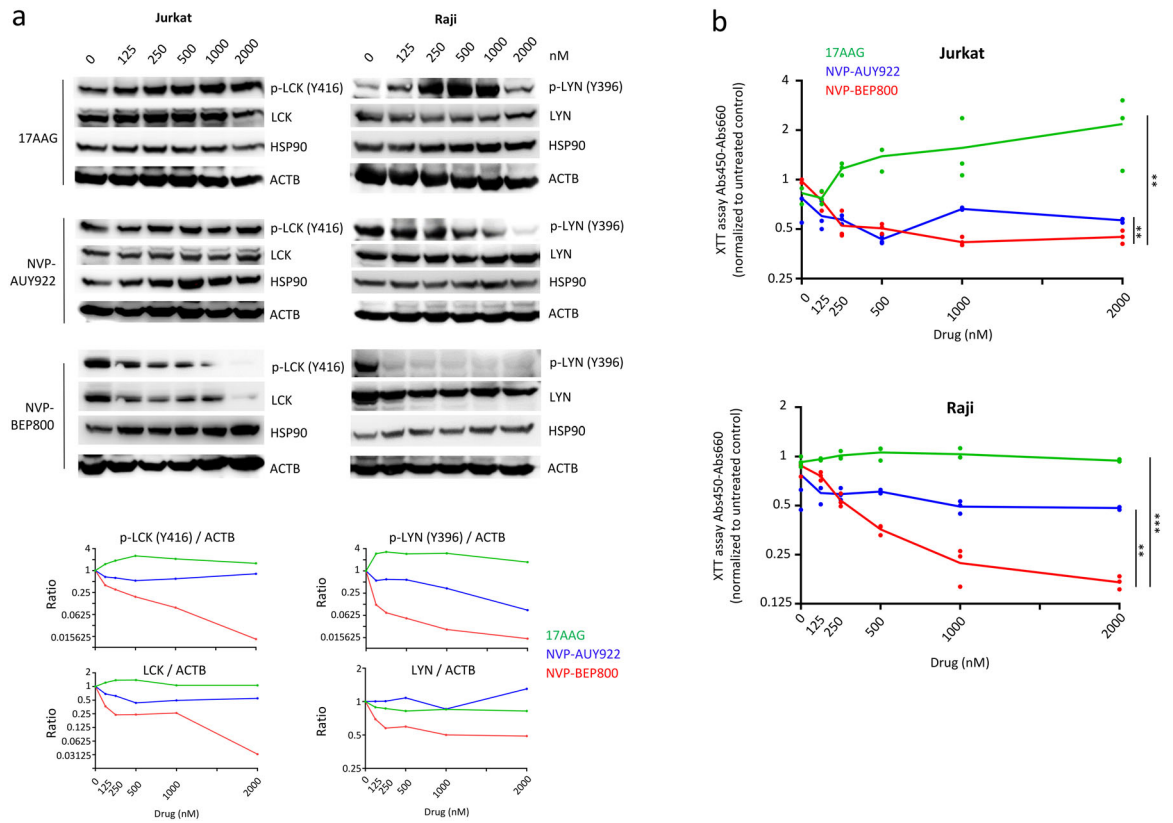

Fig. 1 Sensitivity of T- and B-lymphoid cell lines to NVP-AUY922, 17-AAG and NVP-BEP800. a Western blot shows that in Jurkat and Raji cells, levels of phosphorylated SRC and total SRC proteins are more affected after treatment with NVP-BEP800, compared to NVP-AUY922 or 17-AAG. Western blots after 18 h of treatment are shown on the top panel and quantifications on the bottom panel. **b** XTT viability assay after 48 h of treatment shows that Jurkat and Raji cells are more sensitive to NVP-BEP800, compared to the other two compounds. Data are shown as mean; $n = 3$ biological replicates. *P* value measured by one-way Anova test with Tukey's multiple comparison test; $^{**}P < 0.01$; $^{***}P < 0.001$.

demonstrated different levels of phosphorylated LYN and total amounts of LYN, and the protein levels could be affected by NVP-BEP800 treatment (Supplementary Fig. S2c). With the XTT assay, we also observed that, after NVP-BEP800 treatment, the sensitivity of these cell lines was correlated with the quantity of p-LYN measured by western blot ($R = 0.979$) (Supplementary Fig. S2d). All cell lines expressed the HSP90 chaperone, indicating that sensitivity to the compound was correlated only with p-LYN expression. In addition, NVP-BEP800 which is an ATP-competitive inhibitor specific for HSP90β[48] did not affect the expression levels of neither HSP90α nor β isoforms. Also, NVP-BEP800 did not affect the protein level of the HSP70 chaperone and there was no effect on the BCL2 protein, which is involved in apoptosis (Supplementary Fig. S3).

In conclusion, using T- and B-lymphoid cell lines, we observed that NVP-BEP800, a specific inhibitor of HSP90β, affected the stability of SRC kinases, making them potential clients of the HSP90 protein in lymphoid leukemic cells.

Knockdown of SRC affects response of lymphoid lines to NVP-BEP800

We knocked down LCK or LYN genes' expression, respectively in Jurkat and Raji cells using specific shRNA (shLCK or shLYN), throughout lentiviral infection (Supplementary Figs. S4a and S5a) and observed by western blot specific downregulation of LCK (Fig. 2a) or LYN (Fig. 2b). Specificity of the shRNA for LCK or LYN over other tyrosine kinases was furthermore confirmed (Supplementary Figs. S4b and S5b). Both shLCK and shLYN cells showed a reduction in the percentage of cells in the active phase of division ($Ki67^+$ $7\text{-}AAD^+$) and more cells underwent apoptosis ($Annexin\text{-}V^+$) (Supplementary Figs. S4c and S5c). When we treated shLCK cells or shLYN cells with NVP-BEP800, they showed a significant loss of sensitivity to the compound, 48 h after treatment, compared to shCt control cells ($P < 0.0001$), as measured by XTT viability assay (Supplementary Figs. S4d and S5d). We then analyzed cell growth in vitro during 7 days, shLCK Jurkat cells (Fig. 2c) and shLYN Raji cells (Fig. 2d) showed reduced growth capacity compared to their shCt

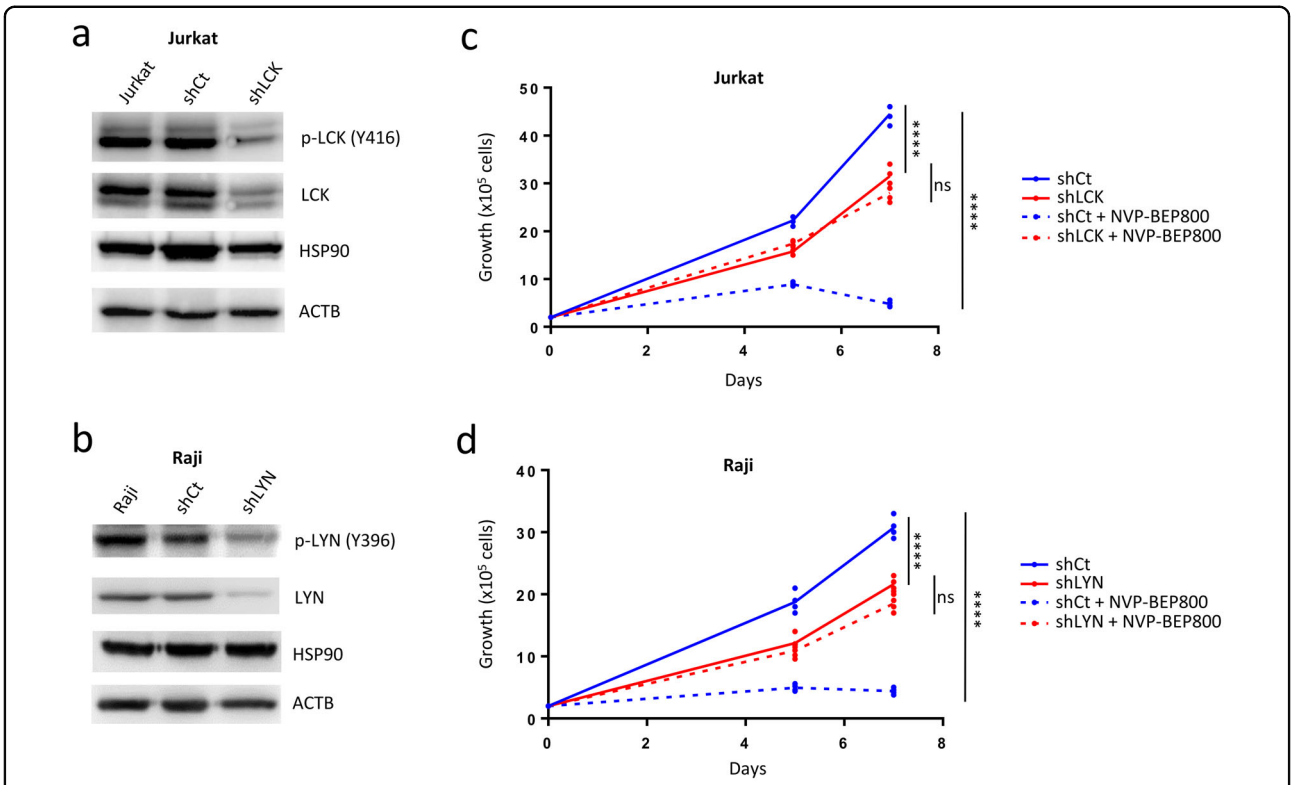

Fig. 2 Downregulation of LCK and LYN kinases inhibits NVP-BEP800 efficiency on T and B lymphoblast cell lines. Western blot shows low levels of expression of p-LCK and total LCK after the viral transduction of Jurkat cells with shLCK (**a**), or p-LYN and total LYN after the viral transduction of Raji cells with shLYN (**b**). Cell growth assay shows no sensitivity of shLCK Jurkat cells (**c**) and shLYN Raji cells (**d**) to NVP-BEP800. 2×10^5 viable cells (trypan blue negative) are seeded. Cells are treated with NVP-BEP800 (1 µM) at days 0 and 5. Data are shown as mean; $n = 3$ biological replicates for T-ALL and $n = 4$ biological replicates for B-ALL. *P* value measured by one-way Anova test with Tukey's multiple comparison test; ****$P < 0.0001$; ns, non-significant.

control cells. Since NVP-BEP800 can target several other clients' proteins[11,52] which went beyond the inhibitory effect of BEP800 on the SRC family of SFK, thus, shLCK Jurkat and shLYN Raji cells treated with NVP-BEP800 showed, albeit slightly, a decrease in growth rate compared to the untreated cells (Fig. 2c, d). However, after treatment with NVP-BEP800, while shCt controls cells were very sensitive to treatment ($P < 0.0001$), shLCK Jurkat cells (Fig. 2c) and shLYN Raji cells (Fig. 2d) were insensitive ($P > 0.05$).

In conclusion, the use of shRNA to knock down LCK or LYN provided evidence that the cytotoxic effects of NVP-BEP800 were mediated by the degradation of SRC proteins.

Sensitivity of primary ALL samples to NVP-BEP800 correlates with expression of SRC

The levels of HSP90 expression in flow cytometry in primary cells isolated from the bone marrow (BM) or peripheral blood (PB) of patients diagnosed with T- or B-ALL was higher than in hematopoietic cells ($CD45^+$) isolated from patients diagnosed with hematological disorders other than ALL (e.g. anemia or thrombocytopenia) (Fig. 3a). We tested the efficiency of the NVP-BEP800 on primary ALL cells in vitro, and observed that both T-ALL cells ($hCD45^+$ $hCD7^+$ cells) and B-ALL cells ($hCD45^+$ $hCD19^+$) were sensitive to the compound, and 2 days after the treatment, a reduction in viability was observed for the 13 T-ALL samples ($P < 0.0001$) and 39 B-ALL samples ($P < 0.0001$) (Fig. 3b). Primary T-ALL cells showed specific expression of LCK, while primary B-ALL cells expressed more LYN (Supplementary Fig. S6). Flow cytometry of primary ALL cells treated with NVP-BEP800 showed a reduction in tyrosine phosphorylation of LCK (p-LCK) in T-ALL cells ($P < 0.0001$) and tyrosine phosphorylation of LYN (p-LYN) in B-ALL cells ($P < 0.001$) (Fig. 3c). We observed that T-ALL and B-ALL cells expressing high levels of p-LCK or p-LYN were more sensitive to NVP-BEP800, and we also noted a correlation ($R = 0.887$ for T-ALL and $R = 0.756$ for B-ALL cells) between the rates of p-LCK or p-LYN measured by flow cytometry before treatment and the percentage of remaining viable cells after treatment (Fig. 3d). No correlation was observed with genetic alterations, and there

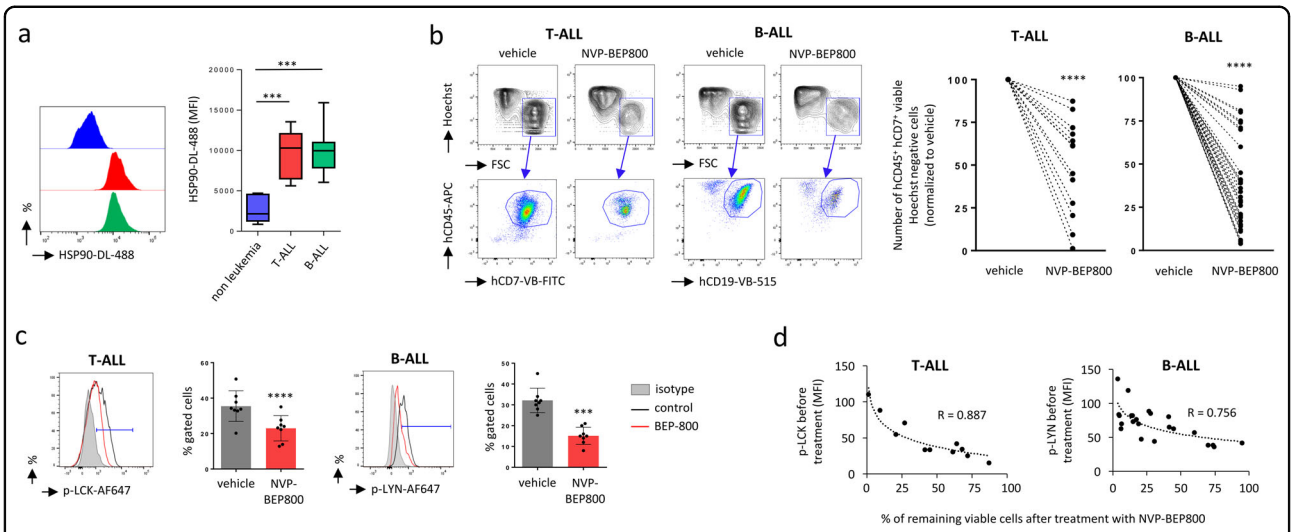

Fig. 3 Primary T-ALL and B-ALL cells are sensitive to NVP-BEP800. a Flow cytometry on primary T-ALL ($CD45^+$ $hCD7^+$, $n = 10$), B-ALL ($hCD45^+$ $CD19^+$, $n = 29$) and non-leukemic ($hCD45^+$, $n = 6$) shows elevated expression of HSP90 for ALL cells. Examples of median fluorescence intensity (MFI) observed by flow cytometry and statistics. Data are presented as median (central line), first and third quartiles (bottom and top of boxes, respectively), and whiskers (extreme values). *P*-value measured by Wilcoxon-Mann-Whitney test; ***$P < 0.001$. **b** Flow cytometry of representative primary ALL cells treated with NVP-BEP800 (1 µM, for 48 h) shows a decrease in the leukemic cell count of T-ALL cells ($hCD45^+$ $hCD7^+$ cells) and of B-ALL cells ($hCD45^+$ $hCD19^+$). Examples of cytometry dataset are shown on the left panel, data are gated on viable Hoechst negative cells. Statistics is shown on the right panel. For quantification, primary cells are fully recorded by flow cytometry. The number of viable primary cells in the group of cells treated with NVP-BEP800 is normalized to control vehicle, for T-ALL ($n = 13$) and B-ALL ($n = 39$). Data are shown as mean ± SD. *P*-value measured by two-tailed paired Student's *t* test; ****$P < 0.0001$. **c** Flow cytometry of representative primary ALL cells treated with NVP-BEP800 (1 µM, for 18 h) shows a reduction of p-LCK in T-ALL ($n = 8$) and p-LYN in B-ALL ($n = 8$) cells. Data are gated on viable Hoechst negative cells, $hCD7^+$ (T-ALL) or $hCD19^+$ (B-ALL). Data are shown as mean ± SD. *P*-value measured by two-tailed paired Student's *t* test; ***$P < 0.001$; ****$P < 0.0001$. **d** The percentage (%) of viable primary cells after treatment with NVP-BEP800 is correlated with the expression of p-LCK or p-LYN, measured by flow cytometry before the treatment, for T-ALL ($n = 11$) and B-ALL ($n = 23$). The logarithmic correlation coefficient R is reported. MFI; median fluorescence intensity.

was no difference in sensitivity detected between children or adults with ALL. However, we observed that sensitivity could correlate with the stage of B-ALL maturation (Supplementary Figs. S7 and S8). We found no correlation between sensitivity to the compound and expression of HSP90 measured by flow cytometry (Supplementary Fig. S9a). Moreover, we found no correlation between the expression levels of HSP90 and SFK proteins (Supplementary Fig. S9b).

In conclusion, primary T-ALL and B-ALL samples showed sensitivity to NVP-BEP800, and this sensitivity was related to their expression of SFK.

NVP-BEP800 affects the SRC signaling pathway in ALL cells recovered from PDX mice

Through the transplantation of primary ALL cells into immunodeficient NSG mice, we generated PDX models to study T-ALL and B-ALL in vivo. By flow cytometry, we detected major expressions of HSP90 and SRC in ALL cells recovered from the BM of T-ALL (Fig. 4a) and B-ALL PDX mice (Fig. 4b). HSP90 is known to regulate the stability of proteins involved in intracellular signaling. Interestingly, fluorescence microscopy revealed that SRC and HSP90 proteins were colocalized in the cytoplasm of T-ALL ($R = 0.91 \pm 0.07$) and B-ALL cells ($R = 0.89 \pm 0.06$) that were recovered ex vivo from the BM of PDX mice (Supplementary Fig. S10). Among the SFK, T-ALL cells expressed more LCK, while B-ALL expressed more LYN, which was confirmed by western blot (Fig. 4c). Previous studies revealed that HSP90 can interact physically with LCK and LYN[46,47]. When SRC kinases were pulled down with specific antibodies, HSP90 was found co-immunoprecipitated in the T-ALL and B-ALL cell lysates, confirming an interaction between HSP90, with LCK in T-ALL and LYN in B-ALL (Fig. 4d).

When cells isolated ex vivo from T-ALL PDX mice were treated with NVP-BEP800, we observed a loss of LCK phosphorylation in its active site (on tyrosine 416) and a loss in the total amount of LCK protein ($P < 0.001$) (Fig. 4e). This kinase, through the calcium influx pathway, was found specific to the regulation of Nuclear factor of activated T cell 1 (NFAT1), which was involved in T-ALL cell survival and proliferation[38]. When inactivated, NFAT1 was phosphorylated (on Serine 54) (Fig. 4e) and left the nucleus to reach the cytoplasm (Supplementary Fig. S11a). Regarding B-ALL cells isolated ex vivo from PDX mice and treated with NVP-BEP800, we observed a loss of LYN phosphorylation in its active site (on tyrosine 396), and a loss in the total amount of

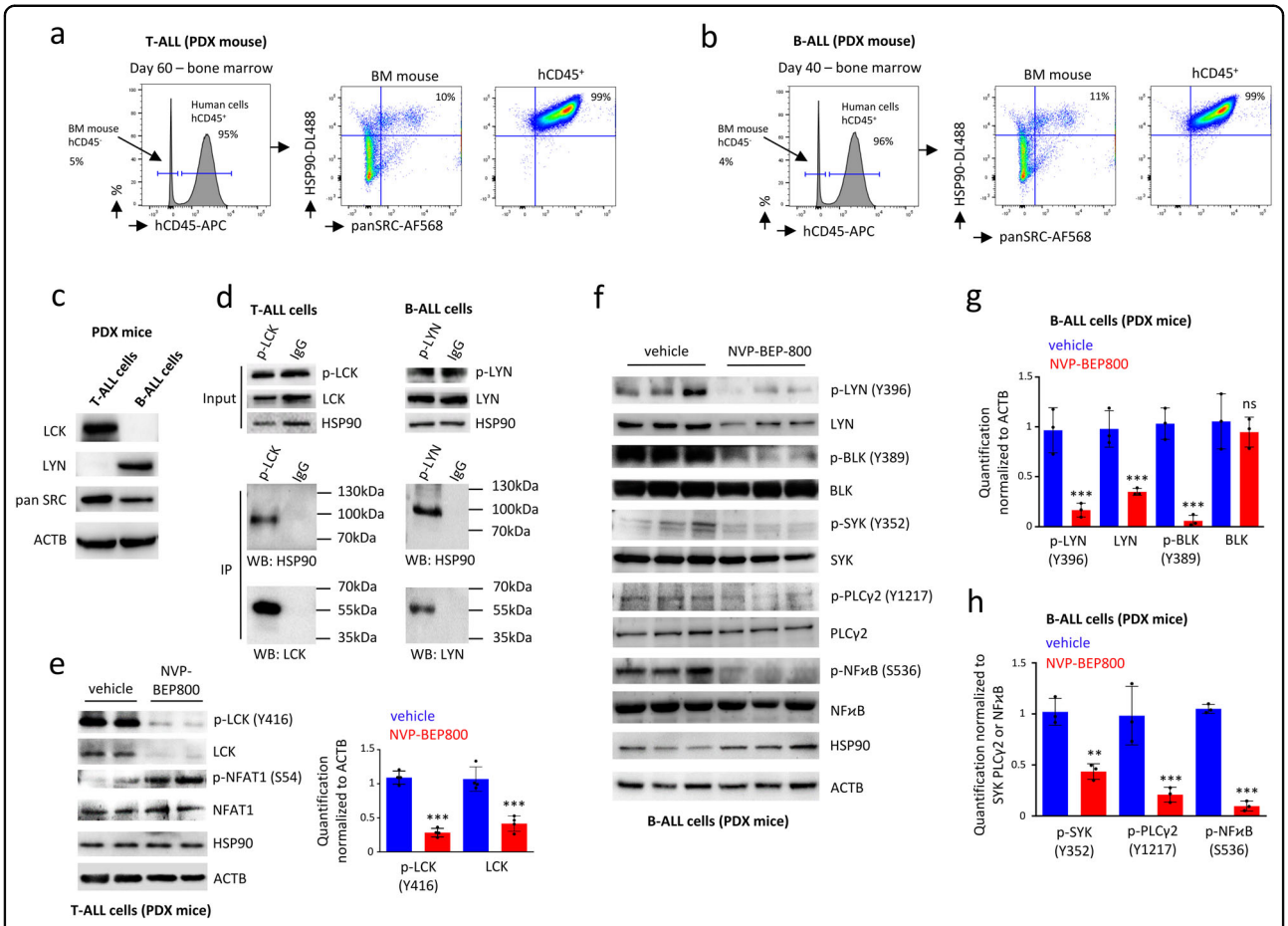

Fig. 4 SRC kinases are client proteins of HSP90. Flow cytometry on BM cells from PDX models of T-ALL (**a**) and B-ALL (**b**), demonstrates that ALL (hCD45^{+}) cells express a high level of HSP90 and panSRC. The anti-panSRC antibody detects murine and human panSRC family kinases, and the anti-HSP90 antibody detects murine and human HSP90. **c** Western blot on ALL cells recovered from PDX mice, shows a high level of expression of LCK in T-ALL, while B-ALL expresses more LYN. **d** Immunoprecipitation shows that LCK binds to HSP90 in PDX T-ALL cells and LYN binds to HSP90 in PDX B-ALL cells. **e** Treatment of PDX T-ALL cells ex vivo for 18 h with NVP-BEP800 at 1 μM affects the stability of the phosphorylated LCK as well as total LCK. The treatment inhibits the transcription factor NFAT1 that becomes phosphorylated. Data are shown as mean ± SD; $n = 4$ biological replicates. *P*-value measured by two-tailed unpaired Student's *t* test; $^{***}P < 0.001$. **f** Western blot shows that treating PDX B-ALL cells with NVP-BEP800 for 18 h, affects the stability of phosphorylated LYN and total LYN. The downstream BCR pathway involving phosphorylation of BLK, SYK, PLCγ2, and NFκB is also affected. **g** Quantification of the western blot shows that among BLK and LYN kinases, only the total amount of LYN is affected following treatment. **h** Quantification of the western blot shows that phosphorylation of SYK, PLCγ2, and NFκB is affected following treatment. Data are shown as mean ± SD; $n = 3$ biological replicates. *P*-value measured by two-tailed unpaired Student's *t* test; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, non-significant.

LYN protein ($P < 0.001$) (Fig. 4f, g). In B-cells, LYN contributed to positive regulation of signaling through tyrosine phosphorylation of the BCR. This role can be assumed by B-lymphocyte kinase (BLK), which can promote B-cells activation through the recruitment of Spleen tyrosine kinase (SYK)[40]. The protein tyrosine kinases, such as LYN, SYK and BLK, and effector enzymes, such as Phospholipase Cγ2 (PLCγ2), played a crucial role in the BCR-induced activation of Nuclear factor κB (NFκB), which was important for the outcome of B-cells[53]. After treatment with the NVP-BEP800 inhibitor, we observed substantial deregulation of the entire signaling pathway, as suggested by the reduced phosphorylation of BLK, SYK, PLCγ2, and NFκB observed with western blot (Fig. 4f, g, h). Upon inactivation, phosphorylation (on Serine 536) was lost and NFκB left the nucleus to reach the cytoplasm (Supplementary Fig. S11b).

We can therefore conclude that the HSP90 chaperone bound SRC kinases in ALL cells and that inhibition of HSP90 through the use of the chemical compound NVP-BEP800 has affected the downstream SRC signaling pathways involved in the proliferation and growth of T-ALL and B-ALL cells.

NVP-BEP800 affects cell cycle and induces apoptosis of ALL cells

SRC kinases were involved in signaling pathways necessary for survival, growth, and maintenance of

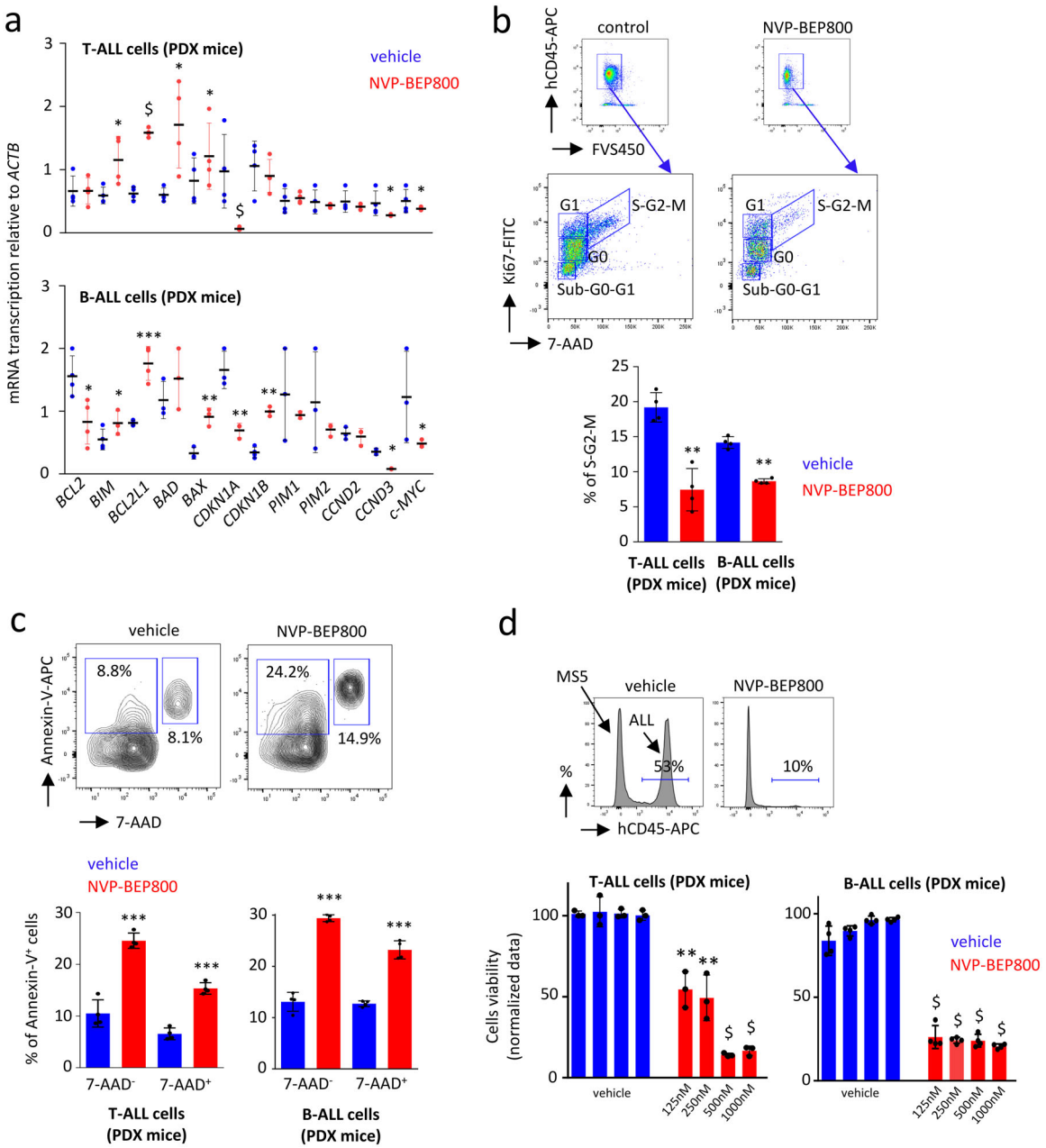

Fig. 5 NVP-BEP800 affects the viability of T-ALL and B-ALL cells. a RTqPCR, performed on T-ALL and B-ALL cells, shows modification in the transcription of genes involved in cell cycle and apoptosis, after treatment with NVP-BEP800 (1 µM) within 18 h. Data are shown as mean ± SD (biological replicates). *P*-value measured by two-tailed unpaired Student's *t* test; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $, $P < 0.0001$; data without statistic are non-significant. **b** A cell cycle study performed by Ki67 staining. **c** An apoptosis study performed by Annexin-V staining. Data shows early apoptotic cells (7-AAD$^-$ Annexin-V$^+$) and late apoptotic cells (7-AAD$^+$ Annexin-V$^+$). Flow cytometry performed on T-ALL or B-ALL cells isolated from BM and treated ex vivo with NVP-BEP800 (1 µM) within 18 h. Data are shown as mean ± SD; $n = 4$ biological replicates. *P*-value measured by two-tailed unpaired Student's *t* test; $^{**}P < 0.01$; $^{***}P < 0.001$. **d** T-ALL ($n = 3$ biological replicates) and B-ALL ($n = 4$ biological replicates) cells are cultured on MS5 support cells for 48 h, under treatment with NVP-BEP800 at increased concentrations (from 125 to 1,000 nM). Ethanol is the vehicle used as control. Flow cytometry is used to quantify ALL cells (hCD45$^+$) after two days of treatment. Data are shown as mean ± SD. *P*-value measured by two-tailed unpaired Student's *t* test; $^{**}P < 0.01$; $, $P < 0.0001$.

T-ALL[36–38] and B-ALL cells[41–43]. To confirm that NVP-BEP800 has an effect on the viability of T-ALL or B-ALL cells recovered from PDX mice, we analyzed the transcription of several genes involved in the cell cycle and apoptosis after treatment (Fig. 5a). NVP-BEP800 increased the transcription of the pro-apoptotic genes

BCL2L1, *BAD*, *BAX*, and *BIM*, and decreased the transcription of *CDKN1A*, a negative regulator of cell levels of p53. Furthermore, treatment with NVP-BEP800 induced the downregulation of *CCND3* and *c-MYC* genes transcription, which are both involved in the cell cycle. Ki67 staining and flow cytometry revealed a marked reduction of T-ALL or B-ALL cells in division (mitosis), following treatment with NVP-BEP800, as demonstrated by the low percentage of cells in the S-G2-M phase (Fig. 5b). Annexin-V staining and flow cytometry showed an increase in the percentage of T-ALL and B-ALL cells undergoing apoptosis after NVP-BEP800 treatment (Fig. 5c), which was furthermore confirmed by increased levels of cleaved Caspase-3 after treatment (Supplementary Fig. S12). When T-ALL and B-ALL cells were cultured on MS5 murine stromal cells for support, we found that the viability of leukemic cells was significantly affected by this treatment (Fig. 5d).

In conclusion, NVP-BEP800 has affected the viability of T-ALL and B-ALL cells ex vivo by dysregulating the SRC kinases involved in cell proliferation and survival.

Activation of LCK or LYN antagonizes the inhibitory effect mediated by NVP-BEP800 on T-ALL and B-ALL cells

SRC kinases were important regulators of TCR and BCR receptors[54]. To confirm the implication of the LCK kinase as the main client of HSP90 in T-ALL, we over activated the TCR pathway via anti-CD3/CD28 monoclonal antibodies. T-ALL cells expressed CD3 and CD28 on the cell surface, as assessed by flow cytometry (Supplementary Fig. S13a). Cross-linking of CD3/CD28 antagonized the ability of NVP-BEP800 to induce complete loss of p-LCK and LCK (Fig. 6a). Signal transduction via CD40 involved activation of LYN kinase and PLCγ2 in B-cells[55], and when these cells were activated via anti-CD40 antibody in vitro they underwent survival[56]. Using flow cytometry, we showed that B-ALL cells expressed CD40 on the cell surface (Supplementary Fig. S13b). On western blot, we found that the cross-linking of CD40 has inhibited the ability of NVP-BEP800 to induce a loss of p-LYN and LYN (Fig. 6b). Although CD3/CD28 stimulated T-lymphocyte proliferation in vitro[57], it induced the apoptosis of T-ALL cells[58]. We observed a slight decrease in T-ALL viability with CD3/CD28; however, the negative effect of NVP-BEP800 on the viability of T-ALL cells was antagonized after cross-linking of CD3/CD28 (Fig. 6c). Additionally, the effect that NVP-BEP800 had on the viability of B-ALL cells was also antagonized after cross-linking of CD40 (Fig. 6c).

In conclusion, we found that an over-activation of the SRC kinase pathways limited the effect of NVP-BEP800 on SRC stability as well as ALL viability, confirming that this compound has affected the SRC signaling pathway involved in the growth of ALL cells.

NVP-BEP800 increases survival of PDX mice developing T-ALL or B-ALL

By flow cytometry, we detected major expressions of HSP90 and SRC for T-ALL and B-ALL cells recovered from the BM of PDX mice, when they were compared to normal murine cells in a BM microenvironment in which leukemic cells were engrafted and expanded (Fig. 4a, b). Therefore, targeting the HSP90 is a good strategy to prevent T-ALL and B-ALL growth in vivo.

When NVP-BEP800 was injected intravenously (i.v.) at 10 mg/kg, we detected a concentration of 5 μM in the plasma as well as in BM, one hour after the injection (Supplementary Fig. S14), and this concentration was approximately five times the half maximal inhibitory concentration (IC50) observed in vitro. Based on this, we investigated to what extent NVP-BEP800 efficiently interfered with leukemia progression in vivo. The first group of mice was injected i.v. with 10 mg/kg of NVP-BEP800 on day 20, 25, and 30 after the transplantation of 100,000 T-ALL cells, while the second group of mice was injected with the vehicle (ethanol). PDX mice treated with NVP-BEP800 survived longer than PDX mice treated with the vehicle ($P < 0.0001$, Fig. 7a). When leukemia progression was followed in PB at day 50 post-transplantation, we observed a reduced amount of leukemic cells ($hCD45^+$ $hCD7^+$) in PB of mice treated with NVP-BEP800 ($P < 0.0001$, Fig. 7b).

Additionally, we developed T-ALL cells that expressed luciferase and green fluorescence protein (GFP) using lentiviral infection of T-ALL cells, and we transplanted these bioluminescent T-ALL cells into mice. Again, the first group of mice was treated with 10 mg/kg of NVP-BEP800 and the second was injected with ethanol on day 20, 25, and 30 after the transplantation of 100,000 bioluminescent T-ALL cells. At day 30 and day 50, we injected luciferin into sleeping mice in order to monitor the location of leukemic cells in living animals, and we observed reduced bioluminescence in the group of mice treated with NVP-BEP800, the difference was evident at day 50 (Fig. 7c). Mice were sacrificed on day 50 post-transplantation. A decrease in bioluminescence was observed in spleens and bones isolated from mice in the treated group (Fig. 7c). When we performed flow cytometry to detect bioluminescent cells that express also GFP, we noted a relevant decrease in T-ALL cells in the PB, BM, and spleens of treated mice (Fig. 7d). Immunohistochemistry on BM and spleen sections stained with an hCD7 antibody revealed a more significant expansion of T-ALL cells in the control group, compared with mice in the treated group (Fig. 7e).

We also used the PDX mice that we developed to investigate to what extent NVP-BEP800 efficiently interfered with B-ALL progression. The first group of mice was injected i.v. with 10 mg/kg of NVP-BEP800 at day 15, 20,

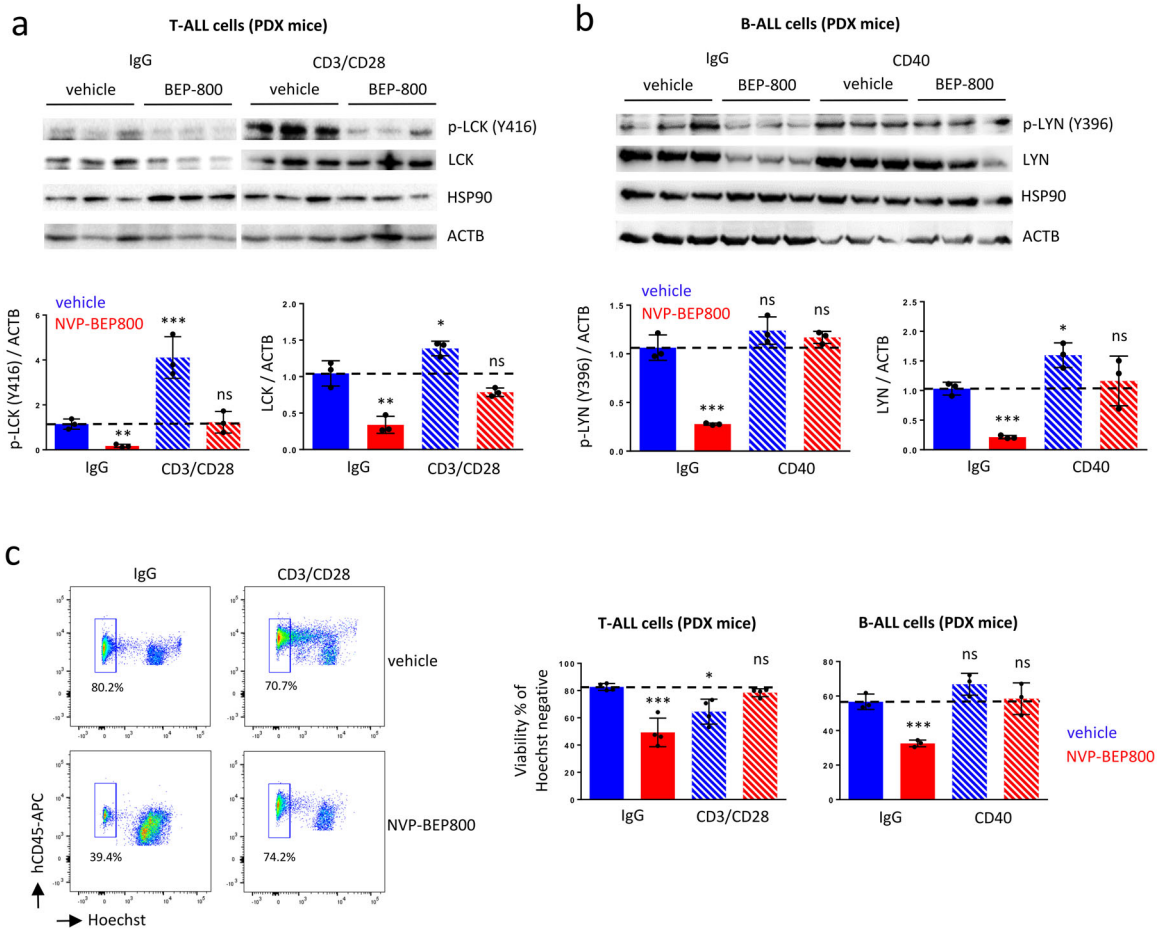

Fig. 6 Activation of LCK or LYN antagonizes the inhibitory effect mediated by NVP-BEP800 on T-ALL and B-ALL cells isolated from PDX mice. a Western blot shows that cross-linking with anti-CD3 (1 µg/ml) and anti-CD28 (1 µg/ml) antibodies (CD3/CD28) to activate the LCK kinase inhibits the effect of NVP-BEP800 and its capacity to reduce levels of p-LCK and LCK. Quantification of p-LCK (Y416) and LCK is normalized to ACTB (bottom panel). Data are shown as mean ± SD; $n = 3$ biological replicates. **b** Activation of the LYN kinase antagonizes the effect of NVP-BEP800. Western blot on PDX B-ALL cells pretreated or not with anti-CD40 (1 µg/ml) antibody (CD40). Quantification of p-LYN (Y396) and LYN is normalized to ACTB (bottom panel). Data are shown as mean ± SD; $n = 3$ biological replicates. **c** Cytometry shows that activation of LCK or LYN antagonizes the inhibitory effect mediated by NVP-BEP800, on T-ALL and B-ALL PDX cells. After treatment with anti-CD3 (1 µg/ml) and anti-CD28 (1 µg/ml) antibodies (CD3/CD28) to activate the LCK kinase or anti-CD40 (1 µg/ml) antibody (CD40) to activate the LYN kinase, ALL cells are treated with NVP-BEP800 (1 µM) for two days and the viability of ALL cells is recorded by flow cytometry after Hoechst staining. Data are shown as mean ± SD; $n = 4$ biological replicates for T-ALL and $n = 3$ biological replicates for B-ALL. In this figure, *P*-value measured by one-way Anova test with Tukey's multiple comparison test; IgG vehicle is used as a reference; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, non-significant.

and 25 after the transplantation of 100,000 B-ALL cells, while the second group of mice was injected with the vehicle. PDX mice treated with NVP-BEP800 survived longer than PDX mice treated with the vehicle ($P < 0.001$, Fig. 8a). When B-ALL progression was followed in PB at day 35 post-transplantation, we observed a reduced amount of leukemic cells ($hCD45^{+}$ $hCD19^{+}$) in mice treated with NVP-BEP800 ($P < 0.0001$, Fig. 8b). We also generated a mouse model to study bioluminescence. After transplanting bioluminescent B-ALL cells into mice, we observed at day 35 a reduced bioluminescence in the group of mice treated with NVP-BEP800, as well as for bones and spleens (Fig. 8c). Flow cytometry of GFP^{+} B-ALL cells confirmed the reduced proliferation of leukemic cells in the BM of mice treated with NVP-BEP800 on day 35 post-transplantation ($P < 0.001$, Fig. 8d). This effect was confirmed by immunochemistry on BM sections, after hCD19 staining to detect B-ALL cells (Fig. 8e).

In the end, it was found that NVP-BEP800 was effective in vivo, and that treatment of PDX mice delayed the development of T-cell and B-cell ALL.

Discussion

Within the cell, HSP90 plays a critical role in the proper folding, assembly, and maintenance of the three-dimensional structures of a variety of proteins, referred

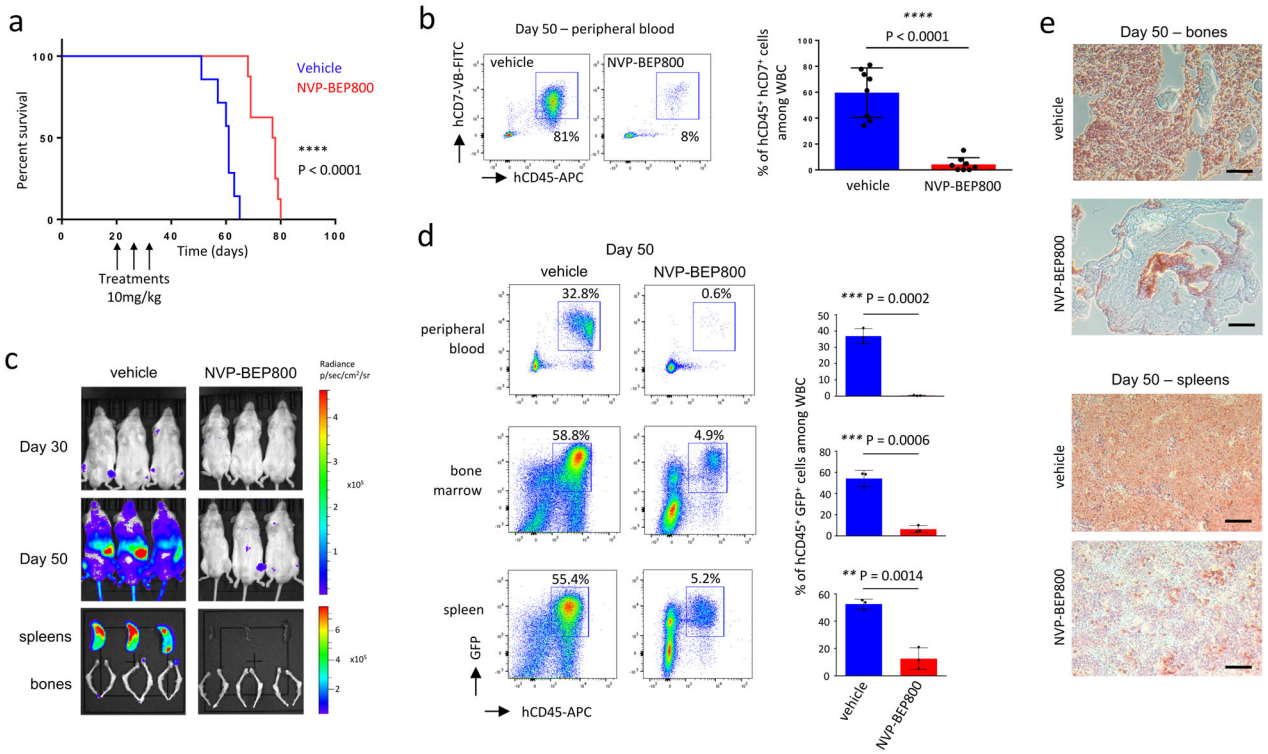

Fig. 7 Treatment with NVP-BEP800 improves the survival of T-ALL PDX mice. a Survival curves show that mice treated with NVP-BEP800 (10 mg/kg) survived longer than mice treated with vehicle, $n = 8$ mice per group. *P* value measured by Mantel Haenszel test. The timing of treatment is shown on the graphic. **b** Percentage of T-ALL cells ($hCD45^+$ $hCD7^+$) is measured by flow cytometry in PB at day 50 of the study, $n = 8$ mice per group. Data are shown as mean ± SD. *P*-value measured by two-tailed unpaired Student's *t* test; $^{****}P < 0.0001$. **c** Mice are transplanted with bioluminescent T-ALL cells and treated with NVP-BEP800. Bioluminescence images ($n = 3$ mice) per group, are taken at days 30 and 50 post-transplantation of bioluminescent T-ALL cells. At day 50, mice are sacrificed and bioluminescence images of their spleens and bones are taken. **d** PB, spleen and BM are analyzed by flow cytometry to detect bioluminescent leukemic cells also expressing GFP and stained with hCD45 antibody. Data shows a reduction of T-ALL cells in all organs in treated mice. Data are shown as mean ± SD; $n = 3$ mice. *P*-value measured by two-tailed unpaired Student's *t* test; $^{**}P < 0.01$; $^{***}P < 0.001$. **e** Immunohistochemistry on bones and spleens sections shows a decrease in the amount of T-ALL cells (brown cells) in mice treated with NVP-BEP800. Data are representative of three mice, magnification ×10, black scale bars represent 200 µm.

to as clients. The molecular chaperone HSP90 is a key member of the cellular proteostasis network, and thus helps protect cells from proteotoxic stress. Cancer cells have up-regulated members of this network, including HSP90, to promote their survival and growth. HSP90 inhibition has been shown to be effective in treating lymphomas[15–17], AML[18–21], CML [26–28], and myeloproliferative neoplasms[24]. Regarding ALL, an interesting study showed that the NOTCH1 signaling status correlated with epichaperome levels and predicted T-ALL cells' response to HSP90 inhibition with the PU-H71 inhibitor[29]. Another study described that NVP-AUY922 led to a degradation of tyrosine kinase 2 (TYK2) signaling and T-ALL apoptosis[30]. In a subset of B-ALL, genetic resistance to JAK2 inhibition was overcome by HSP90 inhibition[31].

In cancers, epichaperone re-wiring altered a plethora of post-translational interactions and many of which converge upon MYC[59]. However, in our PDX models, T-ALL and B-ALL cells did not express MYC (Supplementary Fig. S15a). In several other cancers, HSP90 inhibitors allowed to discover other client proteins of HSP90, such as STAT3 or AKT[11,52]. These client proteins were not involved in the growth and development of leukemic cells in our PDX models, and neither STAT3 nor AKT were phosphorylated in T-ALL and B-ALL cells (Supplementary Fig. S15a, b). Moreover, drugs that inhibit AKT or STAT3, such as MK-2206 or Niclosamide, did not affect the viability of T-ALL and B-ALL cells (Supplementary Fig. S15c).

Several HSP90 inhibitors have undergone clinical trials, but these drugs, which bound to a shared nucleotide pocket in the N-terminal domain, did not differentiate between four different HSP90 family members: HSP90α, HSP90β, GRP94 (Glucose-regulated protein 94 kDa), and TRAP1 (Tumor necrosis receptor-associated protein 1). Therefore, there was a need to identify chemical compounds that were more specific to HSP90β[60]. NVP-BEP800 was found as a potent inhibitor of HSP90β that was 70-fold less effective against other HSP proteins and

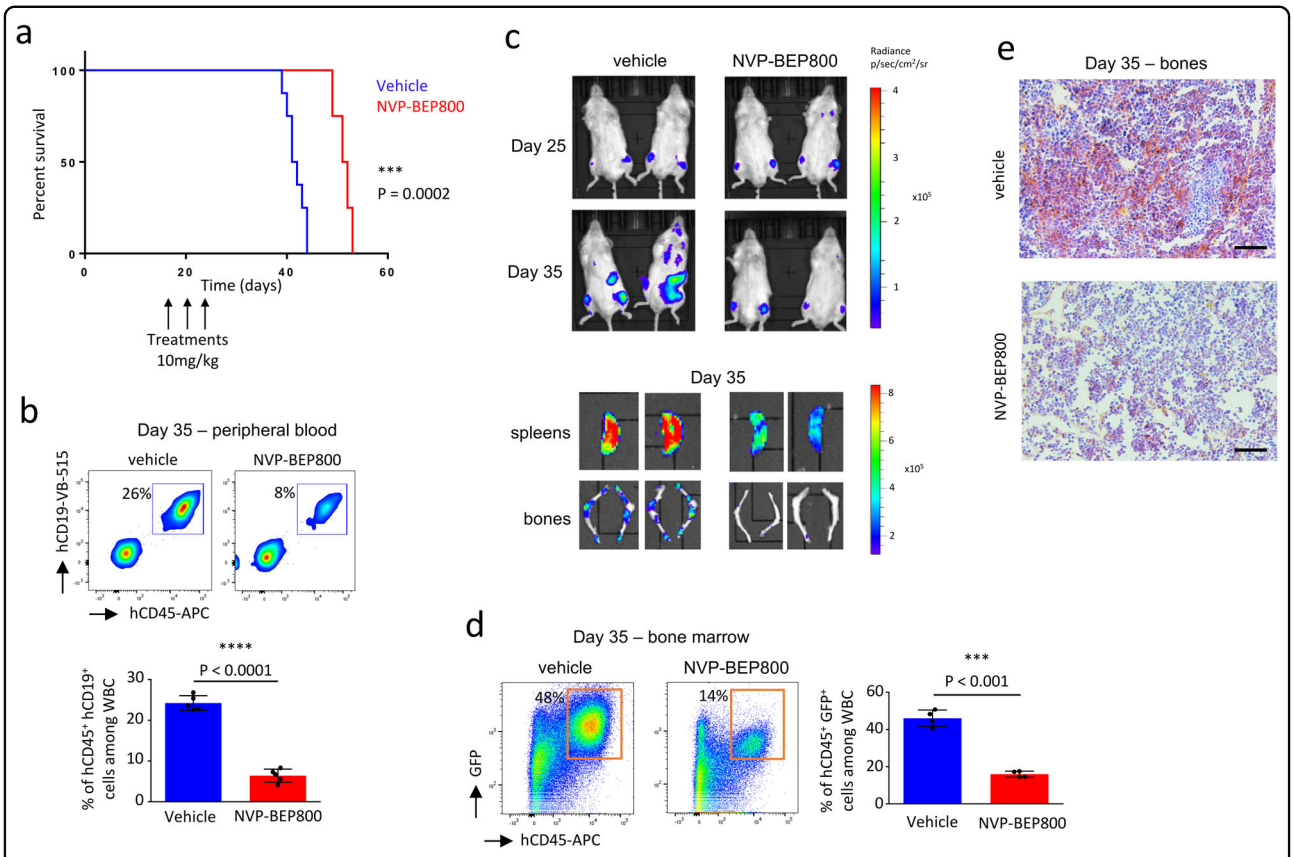

Fig. 8 Treatment with NVP-BEP800 improves survival of B-ALL PDX mice. a Survival curves show that mice treated with NVP-BEP800 (10 mg/kg) survived longer than mice treated with vehicle, $n = 8$ mice per group. *P* value measured by Mantel–Haenszel test; ***$P < 0.001$. The timing of treatment is shown on the graphic. **b** Percentage of B-ALL cells (hCD45$^+$ hCD19$^+$) is measured by flow cytometry in PB at day 35 of the study, $n = 5$ mice per group. Data are shown as mean ± SD. *P*-value measured by two-tailed unpaired Student's *t* test; ****$P < 0.0001$. **c** Mice are transplanted with bioluminescent B-ALL cells and treated with NVP-BEP800. Bioluminescence images of two mice per group, are taken at days 25 and 35 post-transplantation of bioluminescent B-ALL cells. At day 35, mice are sacrificed and bioluminescence images of their spleens and bones are taken. **d** Data show a reduction of B-ALL cells in BM of treated mice, as analyzed by flow cytometry to detect bioluminescent leukemic cells also expressing GFP and stained with hCD45 antibody. Data are shown as mean ± SD; $n = 4$ mice. *P*-value measured by two-tailed unpaired Student's *t* test; ***$P < 0.001$. **e** Immunohistochemistry on bones sections shows a decrease in the amount of B-ALL cells (brown cells) in mice treated with NVP-BEP800. Counterstaining with Giemsa. Data is representative of four mice, magnification ×20, black scale bars represent 100 µm.

many kinases[61]. NVP-BEP800 caused HSP90 dissociation, client proteins degradation and led to growth inhibition or induction of cell death in cancer cell lines[61]. In our study, we discovered that HSP90 was an important regulator of SRC kinases, which were involved in the intracellular signaling pathways necessary for the growth and proliferation of T-ALL and B-ALL cells. Lymphocyte-specific SRC family kinases (SFK) were highly important for both T cells[34–37] and B cells[39,40] proliferation. The inhibition of SRC kinases' phosphorylation, mainly with the use of Bosutinib, Dasatinib, or Saracatinib, was therefore an important strategy for the treatment of T-ALL[36–38] and B-ALL[41–44]. Using an inhibitor of the chaperone that controls the overall level of SRC is likely a good strategy for the development of therapies based on the SRC kinase inhibition in ALL. In our study, we showed that NVP-BEP800 affected phosphorylated SRC and, simultaneously, the total amount of SRC kinase in cells. It seems however that the total LCK protein was more affected than the total LYN protein, this was observed when both cell lines and ALL cells xenografted in NSG mice were treated with NVP-BEP800. We can therefore assume that HSP90 may interact more with the phosphorylated LYN. Previous studies on AML or myeloproliferative neoplasm cells described the efficiency of co-treatments of HSP90 inhibitors with tyrosine kinase inhibitors[22,25]. In our study, we however observed that co-treatment with Dasatinib, a specific inhibitor of SRC phosphorylation, did not increase in vitro the effect mediated by NVP-BEP800 on the viability of cell lines, as well as on T-ALL or B-ALL cells isolated ex vivo from PDX mice (Supplementary Fig. S16). This is probably because NVP-BEP800, by itself, showed a pertinent dysregulation of phosphorylated SFK.

Most studies with NVP-BEP800 were performed in vitro. In these experiments, a decrease in the migration and invasion of lung carcinoma and glioblastoma cells was observed[62], along with apoptosis of myeloma cells cultured on stromal support cells[63], and a reduced proliferation of other tumor cell lines[64]. HSP90 inhibitors, including NVP-BEP800, increased the sensitivity of tumor cells to ionizing radiation[64–66]. In hepatocellular carcinoma, this compound has been found to suppress the vasculogenic networks, which play an important role in tumor malignancy[67,68]. NVP-BEP800 affected the proteasomal degradation of viral HSP90 client proteins, including those required for latency and infectivity of Kaposi sarcoma-associated herpes virus[69]. NVP-BEP800 induced robust antitumor responses on a preclinical xenograft mice model of human breast cancer[61]. Our study showed that NVP-BEP800 has effectively targeted SRC kinases, which should now be considered as novel clients of the HSP90 chaperone. In addition, mice that were treated with NVP-BEP800 survived longer and showed fewer symptoms of leukemia in vivo, confirming that this treatment was effective on both PDX models of T-ALL and B-ALL.

More recent understanding has highlighted that vulnerability of cancer cells to HSP90 inhibitors depends upon pathologic hyperconnectivity within the "epichaperome", composed of chaperone and co-chaperone complexes, this has been characterized for solid cancers[59,70], as well as for T-ALL[29]. In our study, we discovered for the first time that HSP90 can bind and control the stability of SRC kinases in ALL, therefore SFK should be considered as important client proteins involved in the epichaperome for T-ALL and B-ALL. Furthermore, lack of predictive biomarkers of HSP90 inhibitors for selecting patients who would show efficacy versus lack of response remains to be characterized for ALL. In our study, we discovered that patient samples showing high levels of phosphorylated SRC were more sensitive in vitro to the HSP90 inhibitor NVP-BEP800, and this might help to predict the response of ALL to HSP90 inhibition.

While NVP-AUY922 and 17-AAG targeted both HSP90α and HSP90β[49,50], NVP-BEP800 specifically inhibited HSP90β, blocking its N-terminal ATP-binding pocket[48]. Consequently, HSP90 inhibitors that target more precisely HSP90β may have a distinct feature that would favor their clinical development over other HSP90 inhibitors. Previous in vivo studies showed that NVP-BEP800 provided a high degree of flexibility in dose and schedule within the clinical setting, whereas mice in in vivo experiments began to lose body weight when NVP-BEP800 was administered at a dose of greater than 40 mg/kg daily over two weeks[61]. In our experiments, we confirmed the low toxicity of this drug in vivo, by administering three i.v. injections of NVP-BEP800 of 10 mg/kg every five days, and we showed that the drug had no negative effect neither on the development of mice's body weight (Supplementary Fig. S17a) nor on PB hematopoietic parameters (Supplementary Fig. S17b), as well as on mice's viability after monitoring the mice for two months.

Constitutive activation of the SFK has been described as important for the proliferation of cancer cells in AML[71–73]. In the present study, in addition to previous descriptions in T-ALL or B-ALL[36–38,41–44], SRC kinases were also found to be phosphorylated, which attested to the constitutive activation of the SRC kinases for ALL cells, and their importance for the proliferation and growth of ALL cells, in vitro as well as in vivo. An interaction study conducted on HEK293 cells showed that kinases represented the main clients of HSP90, among them the LCK was found[46]. In another work, affinity enrichment of a library of full-length open reading frames allowed to identify LYN kinase interacting partners, among which HSP90 was identified[47]. A TCR-linked multiprotein complex containing HSP90 and LCK has been already described in T-cells[34]. The HSP90-specific inhibitor 17-AAG selectively disrupted kinase-mediated signaling events, including LCK, in normal T-lymphocyte activation[74]. HSP90 has been shown to play a protective role in the regulation of SRC family proteins, as in neutrophils increasing cell survival[75], or in endothelial cells allowing regulation of the vascular endothelial growth factor receptor[68]. HSP90 bound also to LYN in B-chronic lymphocytic leukemia[45]. In our study, through pull-down assays and treatment with an HSP90 inhibitor, we proved that the SRC kinases LCK and LYN were both clients of HSP90, in T-ALL and B-ALL cells, respectively. We found that the sensitivity of ALL cell lines to NVP-BEP800 was dependent on their expression level of SRC rather than HSP90, and all cell lines expressed HSP90 but only the ones expressing SRC were sensitive to the drug. This was confirmed with the use of lentiviral shRNA tools, as the cells lost their sensitivity to NVP-BEP800 when the expression of LCK or LYN was abolished. This was confirmed for both T-ALL and B-ALL primary cells, as a correlation was observed between their sensitivity to NVP-BEP800 and their expression levels of SRC. Remarkably, in our experiments on cell lines and primary samples, no correlation was observed between HSP90 and SRC protein expression levels. In our study, through the use of NVP-BEP800 and since ALL cells expressing high levels of SFK were more sensitive to HSP90 inhibition, we confirmed that SRC kinases were important to maintain the viability of ALL cells.

Altogether, these findings demonstrated that the chaperoning of SRC kinase by HSP90 contributed to the proliferation and growth of T-ALL and B-ALL cells, which provides novel targeting strategies for ALL treatment. Our promising preclinical test results should be further explored, paving the way for future clinical trials.

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Author contributions
R.M. performed majority of the experiments, analyzed data and help in writing the manuscript; J.S., A.G., L.J. helped with experiments; S.B., P.B. provided human samples; P.-S.B. performed in vivo bioluminescence imaging and discussed the data; Z.M., J.-P.P. performed high-performance liquid chromatography and analyzed data; A.G. performed immunohistochemistry; P.-J.F. provided cytogenetic characterization of human samples; F.G. and C.G. discussed the project; R.Q. conceived the study, performed experiments, analyzed data, prepared the figures and wrote the manuscript. The manuscript has been read and approved by all named authors.

Conflict of interest
The authors declare no competing interests.

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HSP90 inhibitor NVP-BEP800 affects stability of SRC kinases and growth of T-cell and B-cell acute lymphoblastic leukemia

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Supplementary Materials and Methods

Cell culture and treatment with NVP-BEP800

The study was conducted on two T lymphoblast cell lines; Jurkat (Clone E6-1, TIB-152, ATCC) and Rpmi-8402 (CRL-1994, ATCC) as well as four B lymphoblast cell lines; BALL-1 (ACC742, DSMZ), Raji (CCL-86, ATCC), Reh (CRL-8286, ATCC) and Daudi (CCL-213, ATCC). All cell lines were cultured in RPMI-1640 media (Dominique Dutscher) supplemented with 10% fetal bovine serum (Dominique Dutscher) and Penicillin-Streptomycin-Amphotericin (PSA, Pan Biotech). Primary T-ALL and B-ALL cells, preserved in diméthylsulfoxyde (DMSO), were frozen in liquid nitrogen, and then cultured in StemMACS media (Miltenyi Biotec) supplemented with PSA. The same was done for T-ALL and B-ALL cells isolated *ex vivo* from the BM of PDX mice. We always used cells freshly isolated *ex vivo* from the BM in our experiments. ALL cells were also co-cultured with MS5 murine stromal feeder cells (ACC-441, DSMZ), in StemMACS media (Miltenyi Biotec) supplemented with PSA. After treatment, cells were trypsinized and the viability of ALL cells was assessed by Hoechst staining and measured by flow cytometry. The hCD45-APC antibody was used to distinguish between human ALL and mouse MS5 support cells. Cells were grown in an incubator at 37°C in a humid atmosphere and 5% CO_2 pressure. For viability, cells were treated for 48 hours with increasing concentrations or a single dose of 1µM with NVP-BEP800 (SelleckChem). For cell cycle activity, apoptosis, cell signaling and transcriptional expression experiments, cells were treated for 18 hours with NVP-BEP800. Ethanol is the diluent, which served as a control “vehicle”. T-ALL cells were treated with anti-CD3 (1µg/ml, BE0001-2, BioXcell) and anti-CD28 antibodies (1µg/mL, BE0291, BioXcell) for TCR activation and B-ALL cells were treated with anti-CD40 (1µg/mL, BE0189, BioXcell) for BCR activation prior to NVP-BEP800 treatments. For viability, cells were treated for 48 hours with NVP-BEP800 or Dasatinib (SelleckChem) alone, or with a co-treatment of both compounds.

Cell viability assay (XTT)

Viability and proliferation of cell lines were determined using the XTT Cell Viability assay (15960972, CyQUANT XTT Cell Viability Assay, Invitrogen) according to the manufacturer’s instructions. Briefly, cells were seeded in a 96-well culture plate at a density of 2×10^5 viable cells (trypan blue negative) in 200µL of media and were treated with increasing concentrations of NVP-BEP800. The culture plates were

incubated for 48 hours at 37°C with a 5% CO_2 atmosphere. After incubation, cells were collected from all the wells and washed with PBS1× (pH 7.4). A mix containing 60µL “XTT Reagent” and 10µL of “Electron Coupling Reagent” was added to each well and the cell plate was incubated for 4 hours. The absorbance was measured at OD 450nm and 660nm with UV-visible spectrophotometer (Biochrom Asys UVM340) using the media as the blank, and the results were determined by the difference in the absorbance values measured at wavelengths of 450nm and 660nm, respectively.

Western blot

Cell pellets were suspended in RIPA lysis buffer (150mM NaCl, 5mM EDTA (pH 8.0), 50mM Tris (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). On cell lysates, OD 620nm was measured to normalize the amount of the loaded sample. An appropriate quantity of protein was supplemented with 5× Laemmli buffer. Targeted proteins were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. Immunoblot was performed with the indicated antibodies, anti-p-NFκB(S536) (1:1000, #3033, Cell Signaling Technology), anti-NFκB (1:1000, #8242, Cell Signaling Technology), anti-p-LYN(Y396) (1:1000, ab226778, Abcam), anti-LYN (1:1000, #2796, Cell Signaling Technology), anti-p-BLK(Y389) (1:1000, PA5-105866, Thermo Fisher Scientific), anti-BLK (1:1000, #3262, Cell Signaling Technology), anti-p-SRC(Y416) (1:1000, #6943, Cell Signaling Technology) was used for p-LCK, anti-pan-SRC (1:1000, #2108, Cell Signaling Technology), anti-LCK (1:1000, #2752, Cell Signaling Technology), anti-p-PLCγ2(Y1217) (1:1000, #3871, Cell Signaling Technology), anti-PLCγ2 (1:1000, #3872, Cell Signaling Technology), anti-p-NFAT1(S54) (1:1000, 44-944G, Thermo Fisher Scientific), anti-NFAT1 (1:1000, 610702, BD Biosciences), anti-HSP90 (1:1000, ADI-SPA-830, Enzo Life Sciences), anti-HSP90α (1:1000, ADI-SPS-771, Enzo Life Sciences), anti-HSP90β (1:1000, ADI-SPA-842, Enzo Life Sciences), anti-HSP70 (1:1000, ADI-SPA-810, Enzo Life Sciences) and anti-ACTB (1:2500, 612656, BD Biosciences). We also used anti-p-STAT3 (Y705) (1:1000, #9145, Cell Signaling Technology), anti-STAT3 (1:1000, #4904, Cell Signaling Technology), anti-p-AKT (S473) (1:1000, #4060, Cell Signaling Technology) and anti-AKT (1:1000, #4691, Cell Signaling Technology), anti-MYC (1:1000, #5605, Cell Signaling Technology), anti-BCL2 (1:1000, Sc-509, Santa Cruz), anti-cleavedCASP3/CASP3 (1:1000, #9665, Cell Signaling Technology). We also used anti-HCK (1:1000, MAB3915, R&D Systems), anti-BTK (1:1000, #8547, Cell Signaling Technology), anti-SYK (1:1000, ab40781, Abcam), anti-ZAP70 (1:1000, #2705, Cell Signaling Technology), anti-JAK3 (1:1000, #8863, Cell Signaling Technology). Appropriate secondary anti-mouse or anti-rabbit antibodies, conjugated with Horseradish Peroxidase were used (1:5000, Cell Signaling Technology). Chemiluminescence was performed (Chemidoc, Bio-Rad), after applying ultra-sensitive enhanced chemiluminescent (ECL) substrate (SuperSignal West Femto Maximum Sensitivity, Thermo Fisher Scientific). Protein sizes were controlled by a protein ladder (Page Ruler Plus Prestained Protein Ladder, Thermo Fisher Scientific), and protein expression levels were assessed by using ImageJ (NIH).

Immunoprecipitation

Cell pellets were reconstituted in RIPA lysis buffer. After preclearing with agarose protein A beads (Thermo Fisher Scientific) for 2 hours, cell lysates were incubated with anti-p-SRC(Y416) (1:200, #6943, Cell Signaling Technology) or anti-p-LYN(Y396) (1:200, ab226778, Abcam) antibodies overnight. The antibody/protein

complex was then pulled out of the sample using protein A-coupled agarose beads. Agarose beads were washed with the buffer four times, reconstituted in RIPA lysis buffer supplemented with 5× Laemmli buffer, and were then heated at 65°C for 10min. Western blot was performed with the same antibodies and procedure described above, in addition to using secondary anti-rabbit or anti-mouse antibodies specific for immunoprecipitation (TruBlot, Rockland).

Flow cytometry and fluorescent-activated cell sorting (FACS)

After tail vein PB sampling from PDX mice, white blood cells were recovered following hemolysis (NH4Cl 150mM, KHCO3 10mM, EDTA 0.1mM, pH 7.4). Bones, tibias and femurs from the two bottom legs were crushed in a mortar and total BM cells were filtered with a sterile cell strainer (70µm). Spleens were also crushed and filtered with the sterile cell strainer in hemolysis solution, and the cells were washed with PBS1×. The development of T-ALL and B-ALL in NSG mice was characterized in PB and BM by flow cytometry using the following anti-human antibodies; anti-hCD45-APC (1:100, 130-110-633, Miltenyi Biotec), anti-CD7-VioBright-FITC (1:100, 130-123-864, Miltenyi Biotec) for T-ALL and anti-CD19-VioBright-515 (1:100, 130-113-175, Miltenyi Biotec) for B-ALL detection. These antibodies were also used to distinguish between human ALL cells and murine MS5 cells *in vitro*. We also used anti-CD28-PE-Vio770 (130-104-189, Miltenyi Biotec) and anti-CD3-PerCP (345766, BD Biosciences) antibodies to analyze the expression of specific markers by T-ALL cells, as well as an anti-CD40 (1:100, BE0189, BioXcell) antibody with secondary anti-mouse-AF488 (1:500, Thermo Fisher Scientific) for B-ALL cells. To study cell cycle and apoptosis, we used anti-Ki67-FITC (1:20, 556026, BD Biosciences), anti-Ki67-BV421 (1:50, 562899, BD Biosciences), 7-AAD (1:20, 559925, BD Biosciences) and anti-Annexin-V-APC (1:50, 550475, BD Biosciences). For intracellular protein staining, anti-HSP90-DyLight-488 (1:200, ADI-SPA-830-488, Enzo Life Sciences) and anti-p-panSRC-AF647 (1:200, 560096, BD Biosciences) were used after permeabilization. On primary ALL cells, we used an anti-p-SRC-AF647 (1:200, 560096, BD Biosciences) antibody after cells' permeabilization. This antibody detects p-LCK, p-LYN, p-HCK, p-FYN and p-YES1, therefore we performed a TaqMan assay to confirm a specific expression of LCK by T-ALL cells and LYN by B-ALL cells. To show that T-ALL and B-ALL cells in PDX mice expressed high levels of HSP90 and SRC, we also used an anti-SRC (1:200, #2108, Cell Signaling Technology) antibody followed by a secondary anti-rabbit-AF568 (1:500, Thermo Fisher Scientific) antibody. After cell surface staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm Plus Fixation/ Permeabilization Kit (BD Biosciences). Viability was controlled with Hoechst (Life technologies). When cells were permeabilized for intracellular staining, a Fixable Viability Stain 450 was used (FVS450, BD Biosciences). Cell subsets were analyzed using a Canto10 or a LSR-Fortessa (BD Biosciences). Cells were sorted on a FACS Aria cell sorter (BD Biosciences) equipped with BD FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software (V10, TreeStar Inc).

Fluorescence microscopy

ALL cells were purified using anti-hCD45 microbeads (130-045-801, Miltenyi Biotec) on an AutoMACS (Miltenyi Biotec). The cells extracted from BM were permeabilized using BD Cytofix/Cytoperm Plus Fixation/ Permeabilization Kit (BD Biosciences) and then stained with anti-HSP90-DyLight-488 (1:200, ADI-SPA-830-488, Enzo Life Sciences) antibody and anti-SRC (1:200, #2108, Cell Signaling

Technology) followed by a secondary anti-rabbit-AF568 (1:500, Thermo Fisher Scientific) antibody. In addition, on BM ALL cells, we used anti-NFAT1 (1:500, 610702, BD Biosciences) and anti-NFκB (1:500, #8242, Cell Signaling Technology) for intracellular staining, followed by a secondary anti-rabbit-AF488 (1:500, Thermo Fisher Scientific) antibody. Hind limb bones were collected, stripped of soft tissue, fixed and decalcified in a specific buffer (#3800400, Decalcifier-I, Leica) for 48 hours, processed and embedded in paraffin. Thick sections were cut from paraffin-embedded samples and used for fluorescent staining with mouse anti-HSP90-DyLight-488 (1:200, ADI-SPA-830-488, Enzo Life Sciences), rabbit anti-hCD7 (1:200, ab109296, Abcam) or rabbit anti-hCD19 (1:200, SAB5500047, Sigma-Aldrich), followed by anti-rabbit AF568 antibody (Life Technologies). ALL cells and bone sections were fixed with ProLong Gold Antifade reagent containing DAPI (P36931, Thermo Fisher Scientific). Images were acquired with an Axio Imager M2 (Zeiss) coupled with an Apotome.2 and processed for studies (Fiji, NIH software).

Immunohistochemistry

Hind limb bones were collected, stripped of soft tissue, fixed and decalcified (#3800400, Decalcifier-I, Leica) for 48 hours, processed and embedded in paraffin. Spleens were fixed in 10% buffered formalin for 48 hours and embedded in paraffin. Thick sections were cut from paraffin-embedded samples and used for immunohistochemistry. We used rabbit anti-hCD19 (1:100, SAB5500047, Sigma-Aldrich) or rabbit anti-hCD7 (1:100, ab109296, Abcam) followed by a secondary anti-rabbit antibody, conjugated with Horseradish Peroxidase (#MP-7401, ImmPRESS HRP anti-rabbit IgG polymer detection kit, Vector laboratories) and substrate (#SK-4800, vector, NovaRED substrate kit, Vector laboratories).

Quantitative reverse transcription PCR

After mRNA isolation with the RNeasy kit (Qiagen) or mRNA organic extraction with Qiazol (Qiagen), Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega) was used to synthesize cDNA. The following TaqMan assays were then used for TaqMan qPCR: BCL2 (Hs00608023), BIM (Hs00708019), BCL2L1 (Hs00236329), BAD (Hs00188930), BAX (Hs00180269), CDKN1A (Hs00355782_m1), CDKN1B (Hs00153277), PIM1 (Hs01065498), PIM2 (Hs00179139), CCND2 (Hs00153380), CCND3 (Hs01017690), c-MYC (Hs00153408), LCK (Hs00178427), LYN (Hs01015819), HCK (Hs01067403), FYN (Hs00941613), YES1 (Hs01080050) and ACTB (Hs01060665) used as an endogenous control. We used TaqMan Gene Expression Master Mix (Applied Biosystems). Experiments were carried out using the Viia7 system (Applied Biosystems).

shRNA lentiviral cloning, production and transduction

Control lentiviral vector (#111170, Addgene) contained shRNA-targeting Renilla luciferase (Ren.713). Lentiviral vectors carrying shLCK or shLYN targeting LCK or LYN genes were generated, following a previously described protocol,[1] by cloning a 97bp shRNA template sequences for LCK; TGCTGTTGACAGTGAGCGAAGGCATCAAGTTGACCATCAATAGTGAAGCCACAG ATGTATTGATGGTCAACTTGATGCCTGTGCCTACTGCCTCGGA and for LYN; TGCTGTTGACAGTGAGCGACAGATTTGTTTTGACAATGTATAGTGAAGCCACAG ATGTATACATTGTCAAAACAAATCTGGTGCCTACTGCCTCGGA into the GFP-(miR-E)-PGK-Puro vector (#111170, Addgene) after removing the Ren.713. All

constructs were verified by sequencing (Genewiz) and lentiviruses were produced in HEK293 cells after transduction with Lipofectamin 2000 (Thermo Fisher Scientific) of the GFP-(miR-E)-PGK-Puro vector, as well as PAX2 (Addgene, #12260) and pCMV-VSV-G (Addgene, #8454) plasmids. After two days, viral supernatants were recovered and six-well plates were incubated 4 hours with retronectin (Takara, Ozyme). Viral supernatants were then spinoculated for 30min at 4,000g. Cells were cultured on these plates for three days in RPMI-1640 media (Dominique Dutscher). Lentiviral transduced cells (GFP^+) were sorted on a FACSAriaIII cell sorter (BD Biosciences). The knockdown efficiency was determined by western blot.

High-performance liquid chromatography (HPLC)

HPLC was first performed on a serial dilution of NVP-BEP800 in PBS1× *in vitro* to establish a correlation between peak areas measured by HPLC and concentration of NVP-BEP800 *in vitro*. Then concentrations in plasma, BM, kidneys and liver were established. One hour after the injection of NVP-BEP800 at 10mg/kg, mice were anesthetized for tail vein bleeding. Plasma was recovered after a centrifugation of PB at 10,000g for 10min. BM was recovered from bones crushed in a mostar, two bottom legs (tibas and femurs) were crushed in 1mL of PBS1×. The volume of BM was estimated at 5µL. Kidneys and liver were crushed in 3 volumes of PBS1×. Following quantification of NVP-BEP800 by HPLC, concentrations in BM, kidneys and liver were calculated, taking into account the dilution in PBS1×. Preparation of standards and extracts; NVP-BEP800 stock solution (5mM in ethanol) was used to prepare eight calibrant standards (0.078 to 10pmol/µL in 50% Acetonitrile). NVP-BEP800 recovery was assessed by mixing 100µL of each standard with 100µL of either PBS, control plasma or control BM samples. Protein precipitation was achieved at -80°C for one hour with 800µL of ethanol. After a centrifugation step (15min at 15,000g, 4°C) supernatants were collected, evaporated to dryness, and suspended in 100µl of 50% Acetonitrile. Solubilized extracts were further centrifuged at 10,000g for 5min, and supernatant were transferred into autosampler vials. For NVP-BEP800 analysis by Ultra-High Performance Liquid Chromatography (UHPLC), we used a DIONEX Ultimate 3000 UHPLC system equipped with a DGP-3600RS pump, a WPS-3000TRS autosampler and a DAD-3000 detector (Thermo-Fisher, USA). NVP-BEP800 standards and samples (10µL) were analyzed using a Poroshell C8 100×2.1mm, 2.7µm column (Agilent Technologies) at a flow rate of 0.6mL/min, 50°C, with a linear gradient of phosphoric acid (0.1%v/v) buffered ultrapure water (solvent A) and acetonitrile (solvent B) as follows: 10% B for 1min, ramped up to 95% in 4min and held at 95% for 3min. Acquisition was performed at 345nm. Instrument control, data acquisition and analyses was performed with Chromeleon version 7.2.9.

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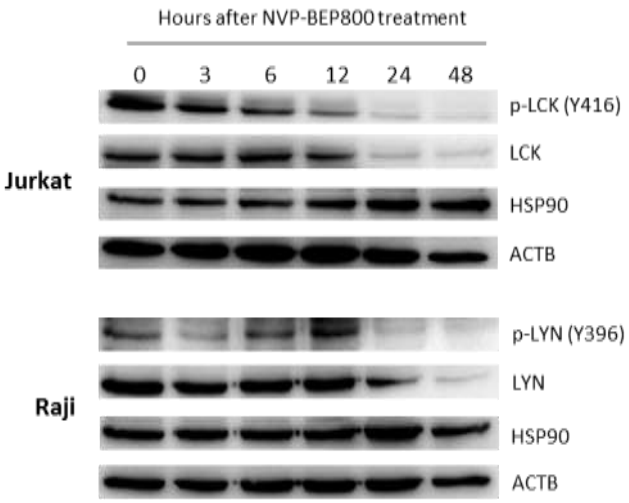
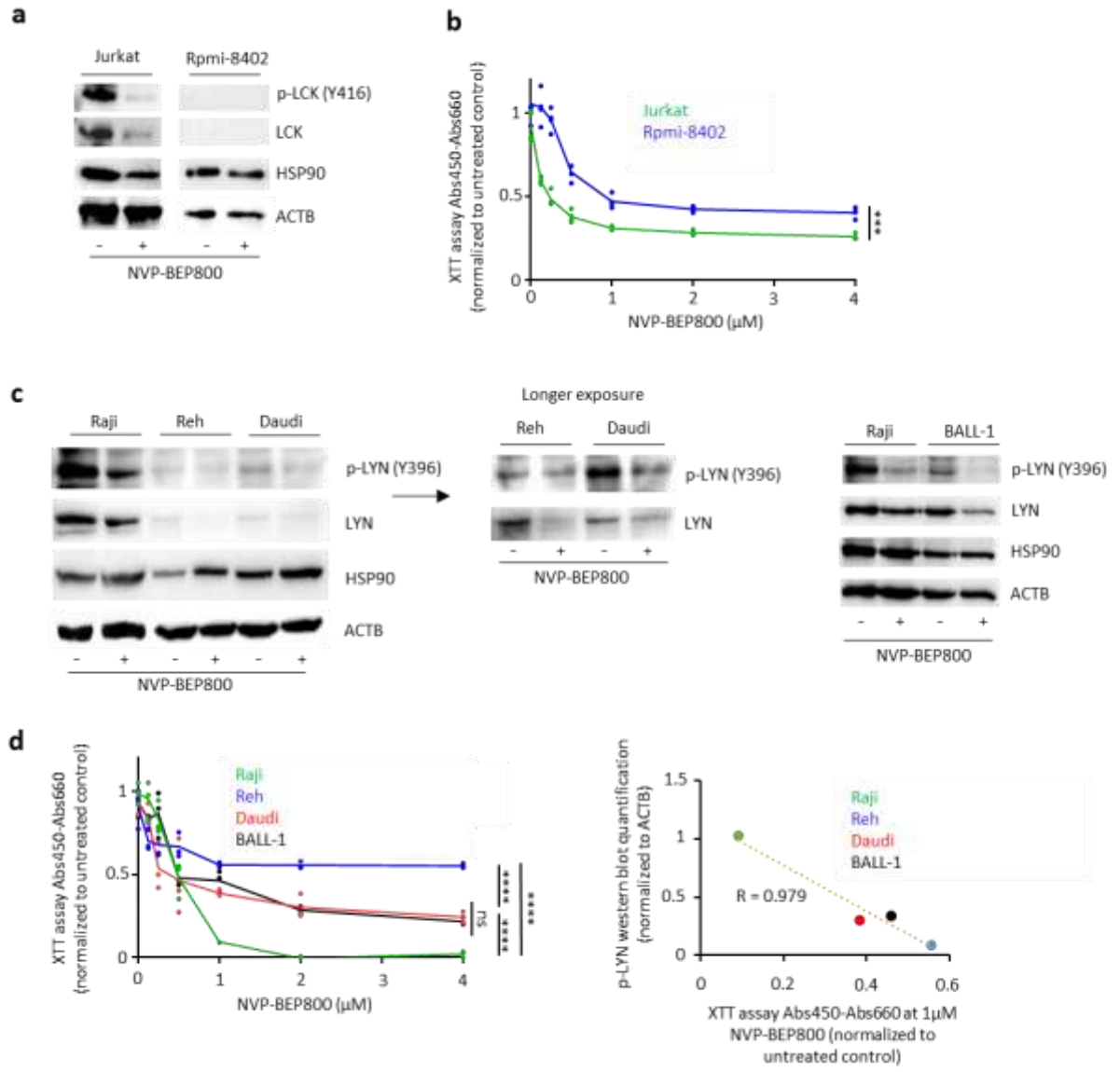
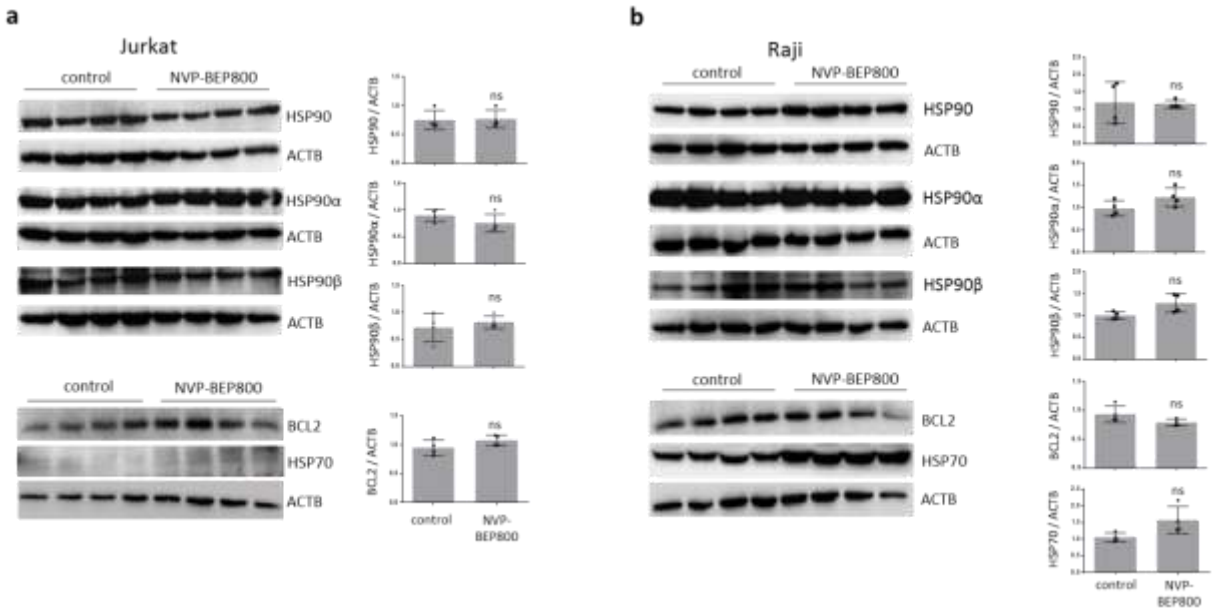
Supplementary Figures



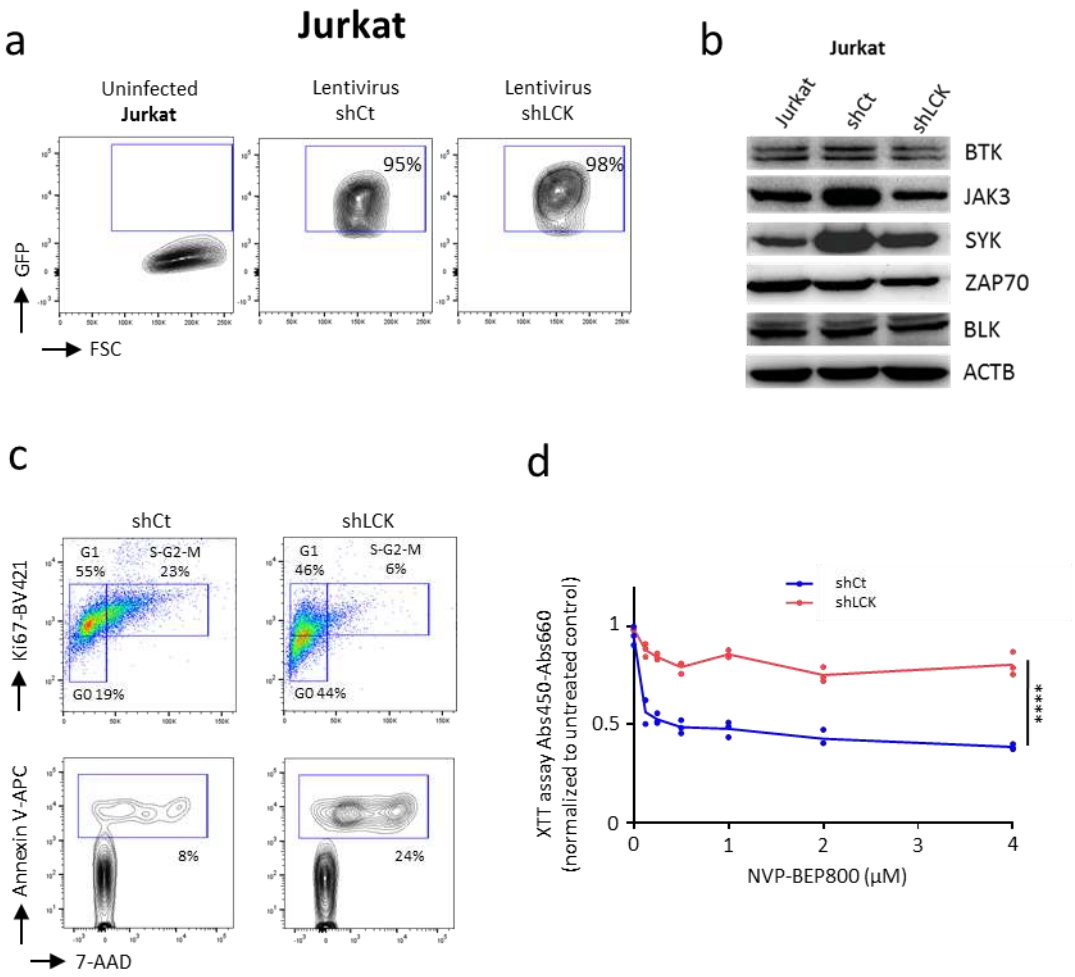
Supplementary Fig. S1. In a time course experiment, western blot showing loss of LCK and LYN expression between 12 and 24 hours after treatment with NVP-BEP800 (1μM) on Jurkat and Raji cell lines.



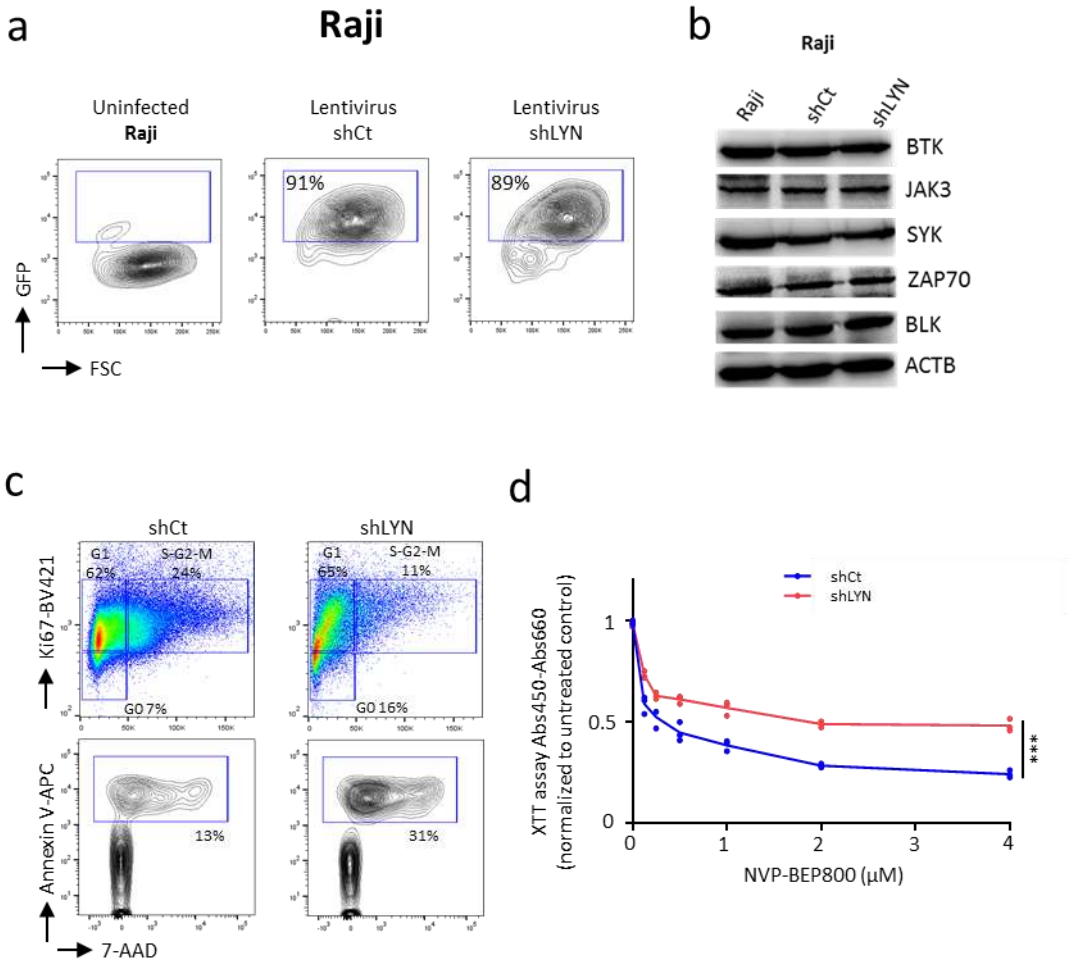
Supplementary Fig. S2. Sensitivity of T and B lymphoblast cell lines to NVP-BEP800 is dependent on SRC expression. (**a**) Western blot shows that the Jurkat cells express more LCK, compared to Rpmi-8402 cells. The LCK kinase is affected after treatment with NVP-BEP800 (1µM for 18 hours). (**b**) The XTT viability assay shows that Jurkat cells are more sensitive to NVP-BEP800, compared to Rpmi-8402 cells, after 48 hours of treatment. Data are shown as mean. P value measured by two-tailed unpaired Student's t-test; ***, P<0.001. (**c**) Western blot shows that out of the four B lymphoblast cell lines, Raji cells expressed high level of p-LYN, while Daudi and BALL-1 cells shows intermediate rates, and Reh cells shows low level. Moreover, cells treated with NVP-BEP800 at 1µM for 18 hours display a reduction in LYN and p-LYN expression. (**d**) XTT viability assay, after 48 hours of treatments, showing that the sensitivity of the four B lymphoblast cell lines to NVP-BEP800 correlates with the expression level of p-LYN. Data are shown as mean (left panel). P value measured by one-way Anova test with Tukey's multiple comparison test; ****, P<0.0001; ns, non-significant.



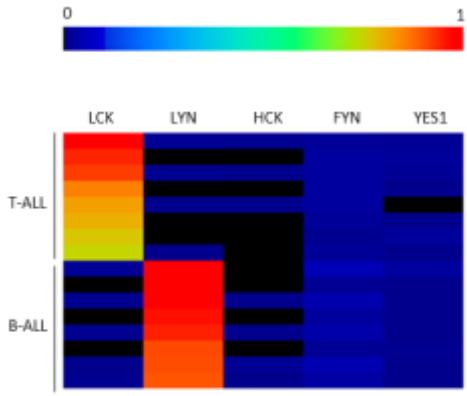
Supplementary Fig. S3. NVP-BEP800 does not affect expression of HSP90, HSP90α, HSP90β, HSP70, or BCL2. Western blot shows that NVP-BEP800 (1μM for 18 hours) does not affect expression of these proteins in Jurkat (**a**) and Raji (**b**) cells. The quantification normalized to ACTB is shown on the right panel. Data shows mean ± SD; n=4. P value measured by two-tailed unpaired Student's t-test; ns, non-significant.



Supplementary Fig. S4. Down regulation of LCK kinase inhibits NVP-BEP800 efficiency on T-ALL Jurkat cells. (**a**) Flow cytometry on Jurkat cells transduced with the shCt or shLCK through lentiviral infection followed by FACS to select GFP^+ cells, as shown in Figure 2a,c. (**b**) Specificity of the shRNAs for LCK (shown in Figure 2a), over other tyrosine kinases. (**c**) Analysis of cell cycle and apoptosis showing a reduction in the percentage of Jurkat cells in the active phase of division ($Ki67^+$ $7\text{-}AAD^+$) and an increase in apoptosis (Annexin V^+) of Jurkat cells expressing shLCK, maintained for 7 days *in vitro*. (**d**) XTT viability assay showing lower sensitivity of shLCK Jurkat cells to NVP-BEP800. 2×10^5 viable cells (trypan blue negative) are seeded and the absorbance is measured to determine viability 48 hours after treatment. Data are shown as mean; n=3. P value measured by two-tailed unpaired Student's t-test; ****, P<0.0001.



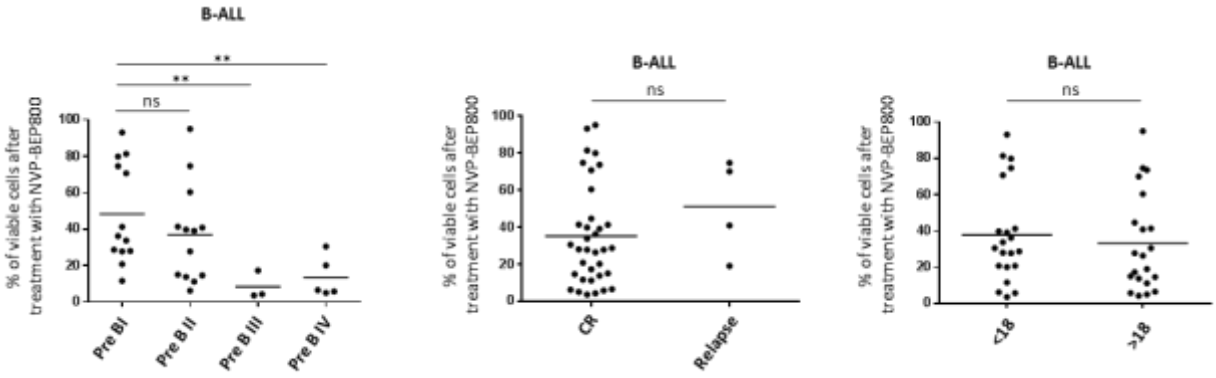
Supplementary Fig. S5. Down regulation of LYN kinase inhibits NVP-BEP800 efficiency on B-ALL Raji cells. (**a**) Flow cytometry on Raji cells transduced with the shCt or shLYN through lentiviral infection followed by FACS to select GFP^+ cells, as shown in Figure 2b, d. (**b**) Specificity of the shRNAs for LYN (shown in Figure 2b), over other tyrosine kinases. (**c**) Analysis of cell cycle and apoptosis showing a reduction in the percentage of Raji cells in the active phase of division ($Ki67^+$ $7\text{-}AAD^+$) and an increase in apoptosis (Annexin V^+) of Raji cells expressing shLYN, maintained for 7 days *in vitro*. (**d**) XTT viability assay showing lower sensitivity of shLYN Raji cells to NVP-BEP800. 2×10^5 viable cells (trypan blue negative) are seeded and the absorbance is measured to determine viability 48 hours after treatment. Data are shown as mean; n=3. P value measured by two-tailed unpaired Student's t-test; ***, P<0.001.



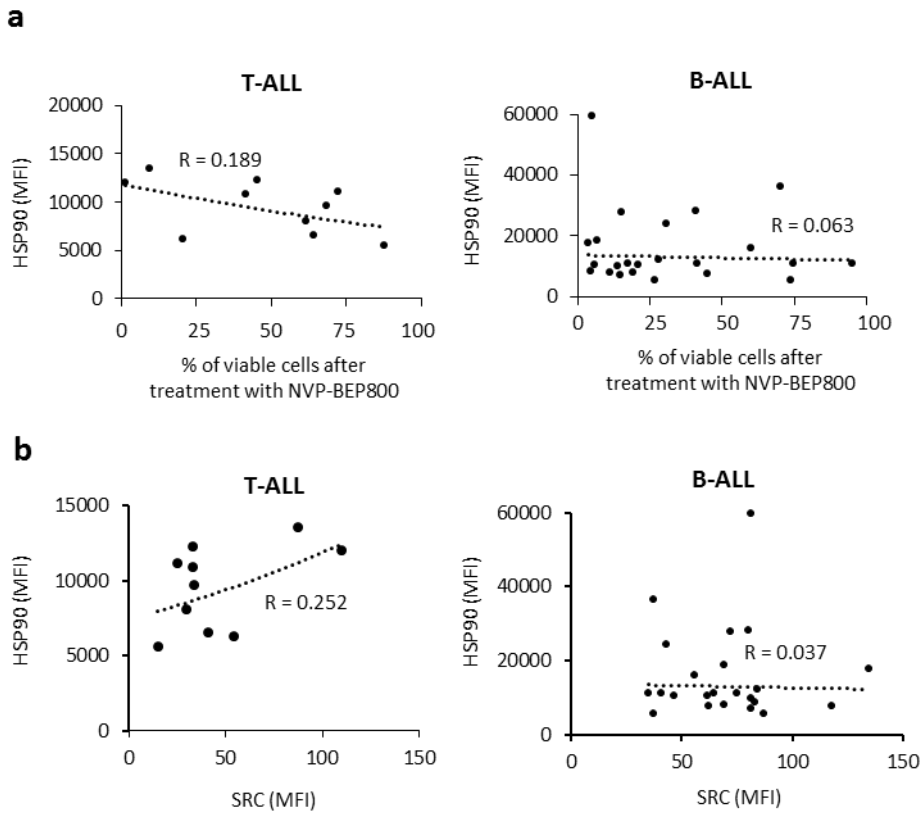
Supplementary Fig. S6. RTqPCR shows specific expression of *LCK* by T-ALL primary cells (n=8 samples) and *LYN* expression by primary B-ALL cells (n=8 samples), among further Lymphocyte-specific SRC family kinases (SFK). Samples tested were the same samples presented in Figure 3C.

T-ALL									
Code	Sex	Age (year)	Source	Blast (%)	Immunophenotype	Viability		Outcome	Genetic
						vehicle	NVP-BEP800		
T2	M	38	BM	80%	T mature	100	1,2	CR	NOTCH1 (m), CDKN2A/B (d)
T9	M	18	PB	98%	Pre/Pro T	100	9,3	CR	NOTCH1 (m)
T5	M	11	PB	82%	T mature	100	20,5	CR	SIL-TAL1 (d)
P2	M	7	BM	92%	Pre/Pro T	100	27,7	CR	TLX3 (d)
T3	M	16	BM	90%	T mature	100	41,4	CR	NOTCH1 (m), CDKN2A/B (d)
T8	M	18	BM	92%	T mature	100	45,0	CR	NOTCH1 (m), SIL-TAL1 (d)
P4	M	11	PB	79%	T mature	100	61,2	relapse	SIL-TAL (d)
T4	M	20	BM	87%	T mature	100	61,5	CR	NOTCH1 (m), PTEN (m)
T1	F	100	PB	74%	T mature	100	64,1	nd	CDKN2A/B (d)
T11	M	49	nd	nd	Pre/Pro T	100	68,1	CR	NOTCH1 (m)
T6	M	40	BM	69%	T mature	100	71,9	CR	nd
P3	M	<18	BM	80%	Pre/Pro T	100	82,6	CR	TLX3 (d)
P1	M	15	BM	85%	Pre/Pro T	100	87,4	CR	TLX1 (d)
B-ALL									
Code	Sex	Age (year)	Source	Blast (%)	Immunophenotype	Viability		Outcome	Genetic
						vehicle	NVP-BEP800		
D3	M	17	BM	66%	Pre B III	100	3,6	CR	BCR-ABL (t)
D5	F	60	BM	84%	Pre B III	100	4,3	CR	BCR-ABL (t)
D14	F	48	BM	65%	Pre B IV	100	5,0	CR	BCR-ABL (t)
D16	F	86	BM	87%	Pre B IV	100	5,8	CR	BCR-ABL (t)
P6	M	5,3	BM	89%	Pre B II	100	6,2	CR	E2A-PBX1 (t)
D13	F	73	BM	72%	Pre B IV	100	6,5	CR	BCR-ABL (t)
D7	M	72	BM	12%	Pre B II	100	11,2	CR	BCR-ABL (t)
P14	M	15	BM	98%	Pre B I	100	11,6	CR	nd
D20	F	62	BM	70%	Pre B II	100	13,8	CR	BCR-ABL (t)
D6	F	28	BM	35%	Pre B II	100	14,6	CR	BCR-ABL (t)
D23	F	63	BM	85%	Pre B II	100	15,0	CR	IGH-CEBPA (t)
D2	F	69	BM	81%	Pre B III	100	17,3	CR	BCR-ABL (t)
D18	M	59	BM	73%	nd	100	19,0	relapse	MLL-AF4 (t)
P1	F	5	PB	95%	Pre B IV	100	20,1	CR	MLL-ENL (t)
D21	M	7	BM	92%	PreB I	100	20,8	CR	nd
D11	M	26	BM	25%	nd	100	26,4	CR	BCR-ABL (t)
P16	F	4,5	BM	94%	Pre B I	100	27,8	CR	hyperdiploidy
D19	F	62	BM	65%	Pre B II	100	27,8	CR	BCR-ABL (t)
P12	M	3	BM	60%	Pre B I	100	28,0	CR	IKZF1 (d), TEL-AML1 (t)
P11	F	2	BM	76%	Pre B I	100	28,7	CR	IKZF1 (d), P2RY8-CRLF2 (f)
D15	M	22	BM	75%	Pre B IV	100	30,5	CR	nd
P10	M	2,5	BM	95%	Pre B I	100	33,8	CR	IKZF1 (d)
P5	M	2	BM	95%	Pre B I	100	36,2	CR	hyperdiploidy
P3	M	7	BM	75%	Pre B II	100	39,1	CR	IKZF1 (d)
P9	F	8	BM	93%	Pre B II	100	39,8	CR	IKZF1 (d), BCR-ABL1 (t)
D10	F	57	BM	45%	Pre B II	100	40,9	relapse	TCF3-PBX1 (t)
P13	F	3	BM	95%	Pre B I	100	41,3	CR	IKZF1 (d)
D9	F	56	BM	23%	Pre B II	100	41,4	CR	BCR-ABL (t)
D12	F	57	BM	61%	nd	100	44,6	CR	BCR-ABL (t)
D4	M	19	BM	26%	Pre B II	100	60,4	CR	BCR-ABL (t)
D17	M	72	BM	45%	nd	100	70,0	relapse	BCR-ABL (t)
P8	M	1,5	BM	85%	Pre B I	100	70,7	CR	IKZF1 (d), P2RY8-CRLF2 (f)
D22	F	68	BM	31%	nd	100	73,6	CR	IGH (a)
D8	M	59	BM	81%	Pre B I	100	74,6	relapse	MLL-AF4 (t), TLX1-TRD (t)
P4	M	4,5	BM	84%	Pre B II	100	74,7	CR	TEL-AML1 (t)
P15	M	4	BM	93%	Pre B I	100	79,9	CR	IKZF1 (d), IGH-DUX4 (t)
P17	M	5	BM	76%	Pre B I	100	81,4	CR	nd
P7	F	11	BM	85%	Pre B I	100	93,1	CR	PAX5 (a)
D1	F	26	BM	73%	Pre B II	100	95,0	CR	PAX5 (a)

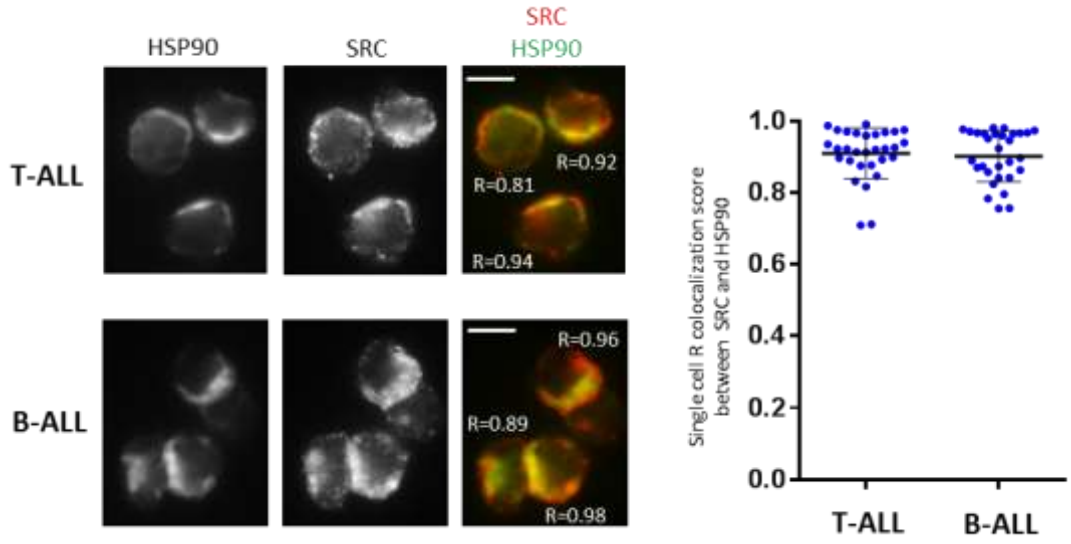
Supplementary Fig. S7. Biological and molecular characteristics of primary T-ALL and B-ALL samples, sorted according to their sensibility to NVP-BEP800 (percentage of viable cells after treatment with NVP-BEP800). CR: cytological remission. (t) translocation, (a) intragenic amplification, (d) deletion, (m) mutation, (f) fusion. BM (bone marrow), PB (peripheral blood). M (male), F (female), cohort of Dijon (D), cohort of Paris (P).



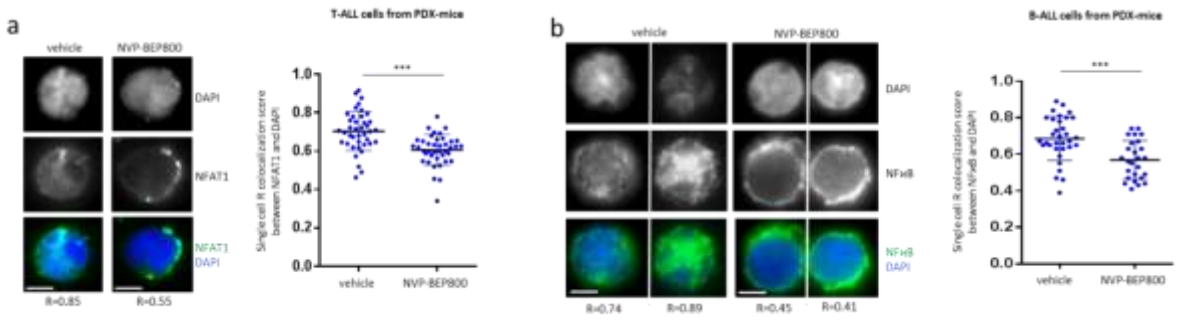
Supplementary Fig. S8. A statistic on primary B-ALL samples, showing the percentage of viable cells after treatment with NVP-BEP800 in different groups; immunophenotype, outcome after treatment (CR: cytological remission) or age of the patients (<18 or >18 years old). P value measured by Mann Whitney test; **, P<0.01; ns, non-significant.



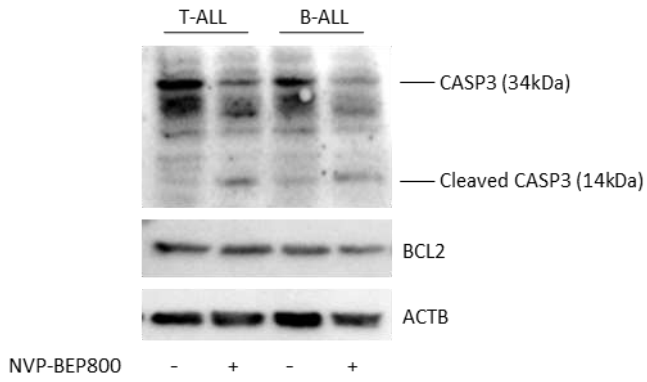
Supplementary Fig. S9. (**a**) A scatter plot showing that the percentage of viable cells after treatment with NVP-BEP800 does not correlate with expression levels of HSP90 measured by flow cytometry, for primary T-ALL and B-ALL samples. (**b**) A scatter plot showing that expression measured by flow cytometry for HSP90 and SRC are not correlated, for primary T-ALL and B-ALL samples. Median fluorescence intensity (MFI).



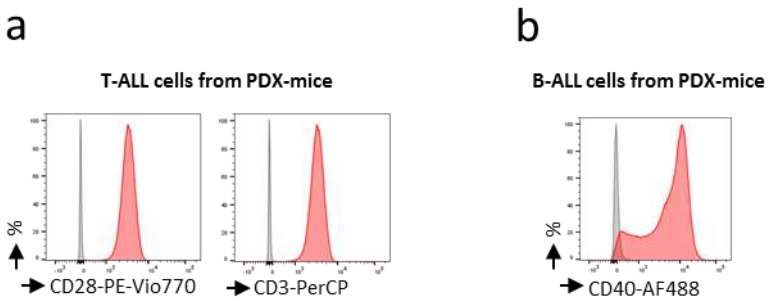
Supplementary Fig. S10. Microscopy showing colocalization between HSP90 and SRC in the cytoplasm for T-ALL and B-ALL cells extracted *ex vivo* using hCD45 microbeads. Example of three cells observed under the microscope on the left panel (Magnification ×63, white scale bars represent 5µm) and colocalisation score (R) observed for single cells (n=30) on the right panel.



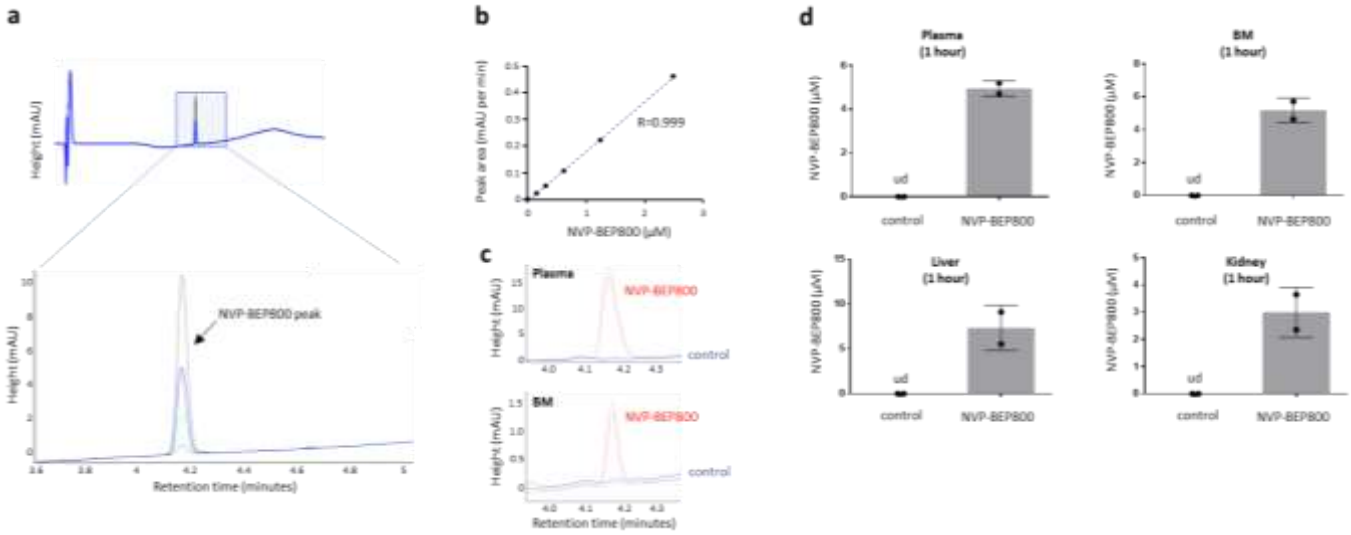
Supplementary Fig. S11. (**a**) Immunofluorescence imaging showing that NFAT1 is excluded from the nucleus after a treatment with NVP-BEP800 (1µM) on T-ALL cells isolated from PDX-mice. Microscopy showing colocalization between NFAT1 and DAPI (nucleus) on T-ALL cells. Example of single cells observed under the microscope on the left panel (Magnification ×63, white scale bars represent 5µm) and colocalisation score (R) observed for single cells (n>30) on the right panel. Data shows mean ± SD. P value measured by two-tailed unpaired Student's t-test; ***, P<0.001. (**b**) Immunofluorescence imaging showing that NFκB is excluded from the nucleus after a treatment with NVP-BEP800 (1µM) on B-ALL cells isolated from PDX-mice. Microscopy showing colocalization between NFκB and DAPI (nucleus) on B-ALL cells. Example of single cells observed under the microscope on the left panel (Magnification ×63, white scale bars represent 5µm) and colocalisation score (R) observed for single cells (n>30) on the right panel. Data shows mean ± SD. P value measured by two-tailed unpaired Student's t-test; ***, P<0.001.



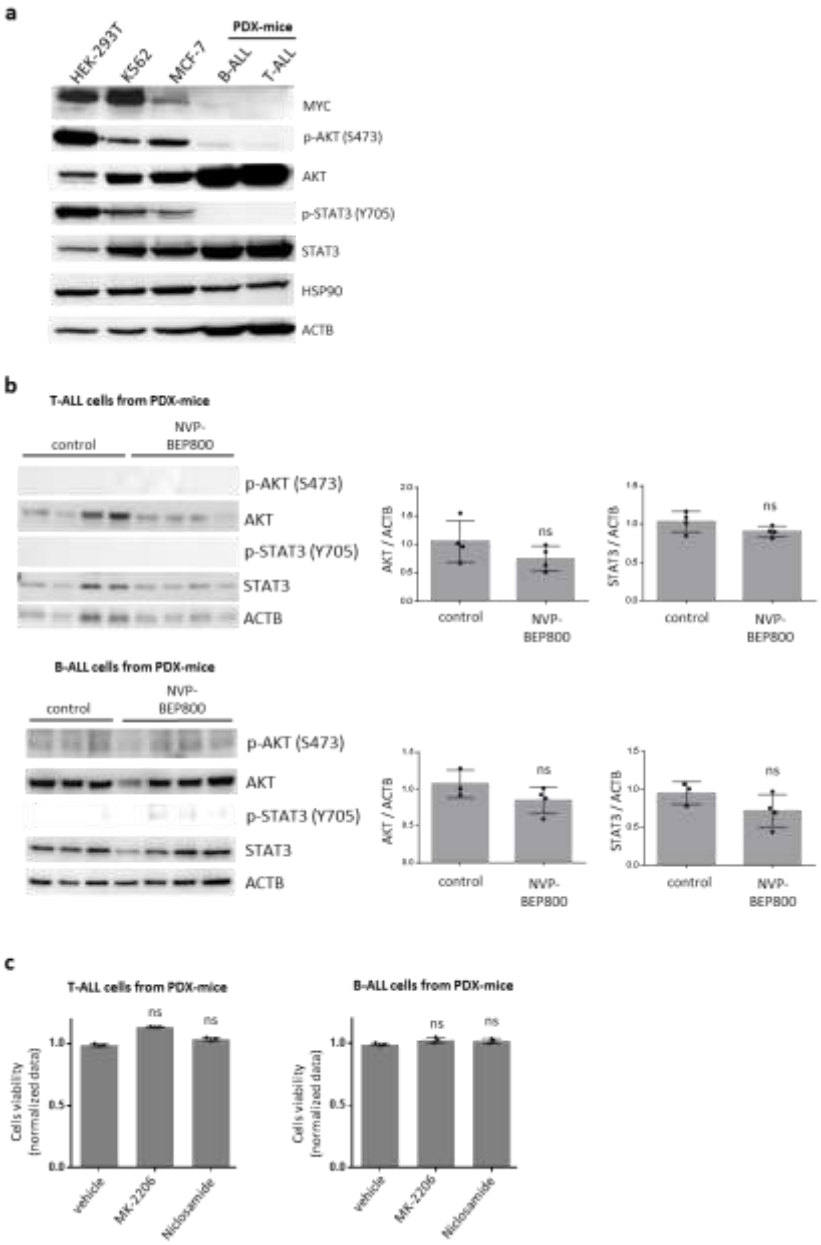
Supplementary Fig. S12. NVP-BEP800 induces apoptosis of T-ALL and B-ALL cells as assessed by western blot showing cleaved Caspase 3 (CASP3) after treatment with NVP-BEP800 (1µM for 18 hours).



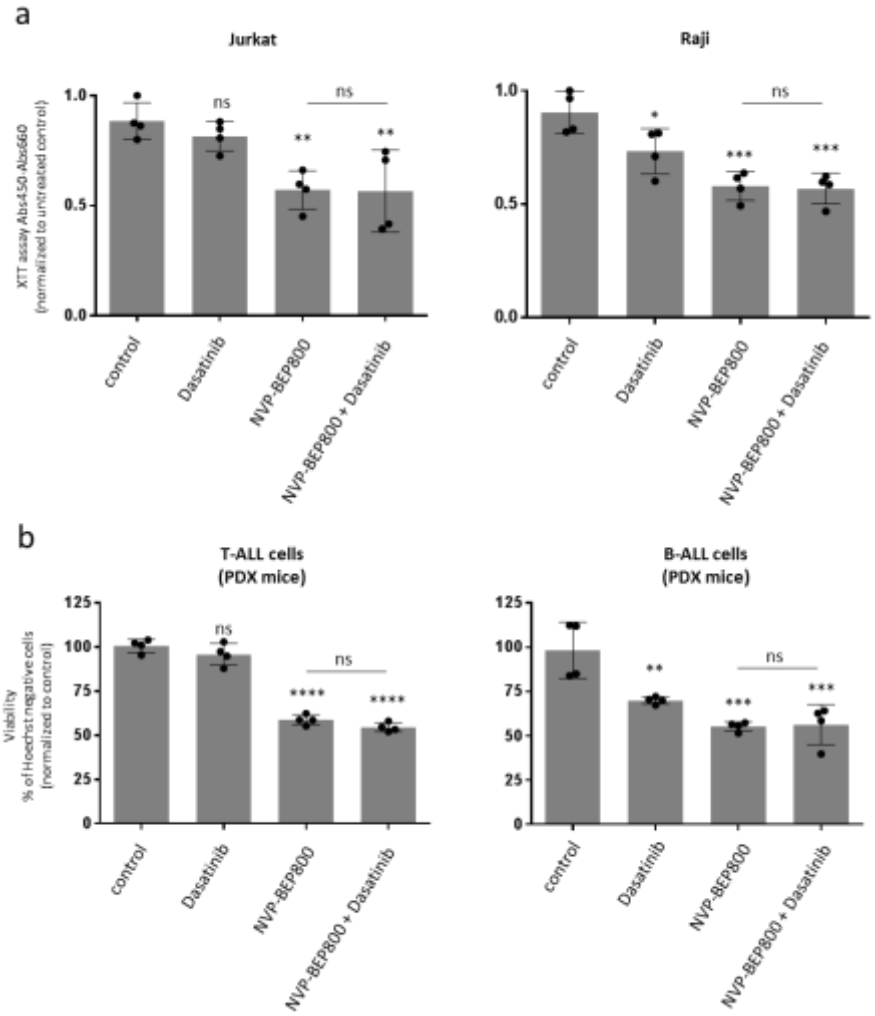
Supplementary Fig. S13. (**a**) Flow cytometry of T-ALL cells recovered from PDX-mice showing expression of CD3 and CD28. (**b**) Flow cytometry of B-ALL cells recovered from PDX-mice showing expression of CD40.



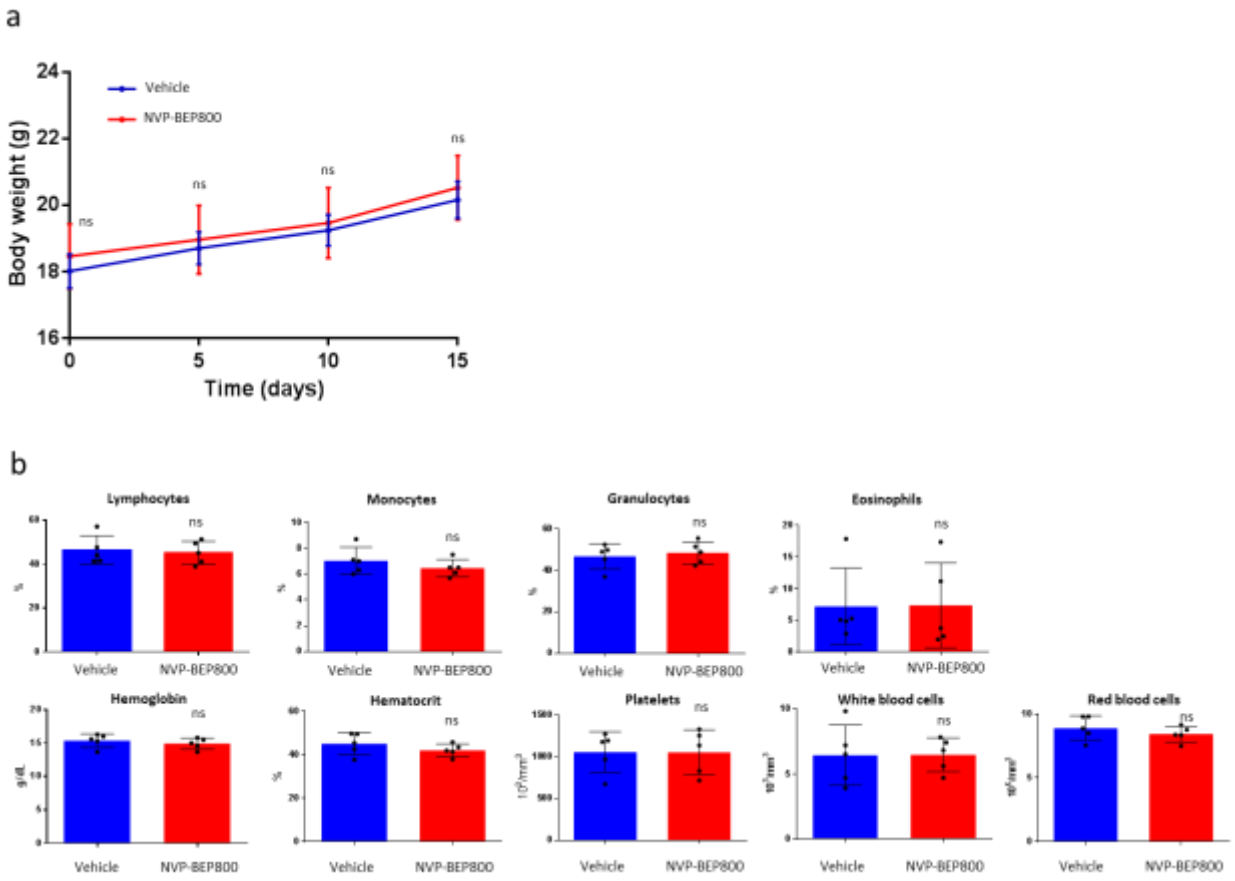
Supplementary Fig. S14. Pharmacokinetic study of NVP-BEP800 *in vivo* by high-performance liquid chromatography (HPLC). (**a**) HPLC results of the serial dilution of NVP-BEP800 in PBS1× *in vitro*. (**b**) Correlation between peak areas measured by HPLC and concentration of NVP-BEP800 *in vitro*. (**c**) Result of the HPLC on plasma and BM samples *in vivo*, mice were analyzed one hour after NVP-BEP800 injection (i.v.) at 10mg/kg. Data shows results from two control mice (blue lines) and two mice injected with NVP-BEP800 (red lines). (**d**) Quantification of NVP-BEP800 *in vivo*, in different tissues; plasma, BM, liver and kidney, one hour after the injection. ud, undetected. Data shows mean ± SD.



Supplementary Fig. S15. T-ALL and B-ALL cells recovered from PDX-mice show no expression of MYC, as well as no activation of STAT3 and AKT pathways. (**a**) Western blot showing that T-ALL and B-ALL cells do not express MYC, as well as phosphorylated STAT3 (Y705) and AKT (S473). Three cell lines were used as positive controls. (**b**) Treatment with NVP-BEP800 does not affect the stability of AKT and STAT3 proteins. The quantification of AKT and STAT3 normalized to ACTB is shown on the right panel. Data shows mean ± SD; n=3-4. P value measured by two-tailed unpaired Student's t-test; ns, non-significant. (**c**) The viability of T-ALL and B-ALL cells recovered from PDX-mice remains unchanged after *in vitro* treatment with inhibitors of AKT (MK-2206, 1µM) or STAT3 (Niclosamide, 1µM). Viability is assessed by flow cytometry. Data shows mean ± SD; n=3. P value measured by one-way Anova test with Tukey's multiple comparison test; ns, non-significant.



Supplementary Fig. S16. XTT viability assay showing that a co-treatment of cells with Dasatinib, a specific inhibitor of SRC phosphorylation, and NVP-BEP800 did not increase the *in vitro* effect mediated by this latter alone. (**a**) Data obtained for Jurkat and Raji cells. 2×10^5 viable cells are seeded and the absorbance is measured to determine viability 48 hours after treatment with Dasatinib (1µM), NVP-BEP800 (1µM) or a co-treatment (1µM each). Data are shown as mean ± SD; n=4. P value measured by one-way Anova test with Tukey's multiple comparison test; *, P<0.05; **, P<0.01; ***, P<0.001; ns, non-significant. (**b**) Data obtained for T-ALL and B-ALL cells, isolated *ex vivo* from the BM of PDX mice. 10^6 cells are seeded and the viability is determined by flow cytometry, 48 hours after treatment with Dasatinib (1µM), NVP-BEP800 (1µM) or a co-treatment (1µM each). Data are shown as mean ± SD; n=4. P value measured by one-way Anova test with Tukey's multiple comparison test; **, P<0.01; ***, P<0.001; ***, P<0.0001; ns, non-significant.



Supplementary Fig. S17. NVP-BEP800 presents no toxicity on NSG mice. (**a**) NVP-BEP800 has no effect on the development of mice's body weight after three i.v. injections of the drug at a dose of 10mg/kg at days 0, 5 and 10. Data shows mean ± SD; n=5. P value measured by two-tailed unpaired Student's t-test; ns, non-significant. (**b**) NVP-BEP800 has no effect on PB parameters, when PB is analyzed at day 15, by using a hemocytometer. Data shows mean ± SD; n=5. P value measured by two-tailed unpaired Student's t-test; ns, non-significant.

III.DISCUSSION, CONCLUSION & PERSPECTIVES

HSP90 inhibitor NVP-BEP800 affects stability of SRC kinases and growth of T-cell and B-cell acute lymphoblastic leukemia

This project has demonstrated important signaling pathways for the proliferation of leukemic cells. We have shown that LCK kinase is involved in the proliferation of T-ALL cells and LYN kinase is involved in the proliferation of B-ALL cells. Several studies have reported that HSP90 levels are elevated in CML and AML cells (**Khajapeer K et al. 2015; Flandrin P et al. 2008** and **Hazza S et al. 2014**) and that expression of HSP90 in patients with ALL was significantly higher than in controls (**Hacihanefioglu A et al 2010** and **Pawlik-Gwozdecka D et al.2020**). Several studies have reported the regulation of LCK and LYN by HSP90 and the interaction between them (**Trentin L et al. 2008; Giannini A and Bijlmakers MJ. 2004)** and in our turn, we have proved these interactions.

Moreover, based on the information that showed that HSP90 inhibition destabilizes the kinase, leading to its subsequent degradation and to a reduction in pro-growth signals in general (**Lerdrup M et al. 2006** and **Trepel J et al. 2010**) and given that a number of clinically relevant HSP90 inhibitors currently exist, the concept of targeting HSP90 as a way to broadly inhibit kinase activity in cancer worth constant consideration (**Whitesell L and Lindquist S. 2005; Lu X et al. 2012; Schwartz H et al. 2015**).

The purpose of this project was to test the HSP90 inhibitor, NVP-BEP800, in order to study its ability to deactivate SFK clients of HSP90 in ALL and to prevent the development of leukemia. Our research showed that NVP-BEP800, affects the viability of T-ALL and B-ALL cells in vitro and in vivo by inhibiting the phosphorylation of LCK and LYN in these cells, therefore, these results allow us to consider a new therapeutic strategy to treat ALL. In this part, our findings are discussed, opening the way for new perspectives.

This research is not the first to highlight the importance of HSP90 inhibitors and SRC TK inhibitors (TKIs) in the treatment of solid cancers and leukemia. In fact, many HSP90 inhibitors like ICPD47 and ICPD6 demonstrated anticancer activity against colorectal, osteosarcoma and cervical cancer (**Daunys S et al. 2019**). Other HSP90 inhibitors like Ganetespib, Alvespimycin and NVP-AUY922 have been shown to be effective in treating various types of leukemia (**Akahane K et al. 2016** and **Lancet J et al. 2010**). In the other side, SRC inhibitors like Saracatinib and Dasatinib have been shown to have a high ability to affect ALL cells proliferation by inhibiting LCK (**Serafin V et al. 2017** and **Buffière A et al. 2018**) and LYN (**Xu Y et al. 2005** and **Foa R et al. 2011**).

1. Expression of HSP90 in acute lymphoblastic leukemia.

Heat-shock protein 90 is an abundant molecular chaperone that plays a vital role in the proper folding, assembly, and maintenance of the three-dimensional structures of a variety of proteins, referred to as clients, involved in apoptosis, survival and growth pathways (**Chiosis G et al. 2004**). HSP90 has been shown to be overexpressed in a number of solid cancers and leukemia, in addition to a large body of evidence supporting the important role of HSP90 in promoting cancer cell survival and growth (**Isaacs J et al. 2003; Whitesell L and Lindquist S. 2005**).

We assessed HSP90 expression levels by flow cytometry, in primary cells isolated from bone marrow (BM) or peripheral blood (PB) of patients diagnosed with T- or B-ALL, and in hematopoietic cells isolated from patients diagnosed with hematological disorders other than ALL. HSP90 was 4-5 highly expressed in ALL cells compared to control cells, and this is consistent with previous studies indicating that cancer cells express 2 to 10 times higher levels of HSP90 intracellularly, when compared to normal cells (**Solárová Z et al. 2015**). As I mentioned above, studies have reported that HSP90 levels are elevated in CML and AML cells, and that these elevated levels of HSP90 could serve as prognostic marker in these types of leukemia (**Zackova M et al. 2013**). Scientists found that HSP90 is overexpressed in B-CLL compared to the resting B cells (**Trentin L et al. 2008** and **Guo A et al. 2018**). Other studies reported that the strong expression of HSP90 in patients with ALL was associated with a low survival rate **(Hacıhanefioglu A et al. 2011**). Moreover, studies reported that plasma HSP90 was validated as a soluble biomarker of ALL, useful for earlier detection of leukemia engraftment (**Milani M et al. 2015)**. Therefore, based on what has been found above, HSP90 expression inhibition could be a suitable therapeutic approach for leukemia compared to conventional chemotherapies since it is target-specific and can have a low toxicity profile.

HSP90 has been shown to correlate with an increased malignant phenotype in breast cancer (**Pick E et al. 2007**), gastric cancer (**Zuo D et al. 2003**) and lung cancer (**Gallegos Ruiz M et al. 2008**). However, the expression of HSP90 was associated with good prognosis in endometrial cancer (**Ciocca D and Calderwood S. 2005**), whereas expression of HSP90 was seen to be of no prognostic significance in ovarian cancer (**Elpek G et al. 2003**) and salivary gland tumors (**Vanmuylder N et al. 2000**). In our study, HSP90 expression measured by flow cytometry was not significantly correlated with survival nor with the sensitivity to NVP-BEP800, which might be due to either the size of our sample or the follow-up interval. No correlation was observed with genetic alterations, and there was no difference in sensitivity detected between children or adults patients

with ALL. However, we observed that sensitivity to treatment could correlate with the stage of B-ALL maturation but not with the stage of T-ALL maturation.

2. The functional roles of HSP90 and the effect of its inhibition in acute lymphocytic leukemia

HSP90 is overexpressed in cancer cells where it is essential for the stability and function of a range of proteins associated with the six hallmarks of cancer. These include proteins involved in cell survival and proliferation AKT, SRC, FLT3, CDK4, CDK6, STAT3, telomerase, MEK, RAF, HIF1, and BCR-ABL (**Hanahan D et al. 2000; Maloney A and Workman P. 2002**). HSP90 inhibitors affect conformational maturation of the complexed-clients and influence the function and degradation of its client proteins, in different oncogenic signaling pathways (**Zhang F and Burrows H. 2004**). HSP90 inhibition has been shown to be effective in treating lymphomas (**Ishikawa C et al. 2016; Jacobson C et al.2016** and **Taniguchi H et al. 2014**), AML (**Katayama K et al. 2018; Lazenby M et al. 2015; Lancet J et al. 2010**), CML (**Khajapeer K and Baskaran R. 2015; Bhatia S et al. 2018** and **He et al. 2016**) and myeloproliferative neoplasms (**Hobbs G et al. 2018**). Among the mechanisms involved in ALL progression, a study showed that the NOTCH1 signaling status controls the levels of chaperone/co-chaperone complexes in T-ALL and predicts the significant response of T-ALL patient samples to HSP90 inhibitor PU-H71. Another study found that TYK2 tyrosine kinase signaling through its downstream effectors mediates the abnormal survival of T-ALL cells. The authors showed that pharmacological inhibition of HSP90 with NVP-AUY922, leads to a rapid degradation of TYK2 and apoptosis in T-ALL cells.

Another work showed that inhibition of HSP90 by IPI504, causes BCR-ABL protein degradation, decreased leukemia stem cell count , and suppression of the initial B-ALL clones that may help prevent the transition of CML to advanced B-ALL induced by *BCR-ABL*-T315I mutation (**Peng C at al. 2007**). In addition, HSP90 inhibitors were effective against *CRLF2* rearrangement B-ALL and other *JAK2*-driven cancers with genetic resistance to JAK enzymatic inhibitors, as they prolonged the survival of mice xenografted with primary human *CRLF2*-rearranged B-ALL cells (**Weigert O et al. 2012**).

Not long ago, it has been found that under conditions of stress, such as MYC-fueled metastasis, the chaperome becomes reconnected to form with HSP90 and HSC70, “the epichaperome”, which can function as a network to enhance cellular survival, regardless of tissue of origin or genetic background (**Rodina A et al. 2016**). Although this epichaperome is present in over half of all cancers tested, however it is not found in our PDX models since T-ALL and B-ALL cells did not

express MYC.

In many other cancers, HSP90, through its interaction with the PI3K/AKT and STAT3/STAT5 transcription factor pathways, can be crucial both for the tumorigenic properties of cancer cells (cell proliferation, survival) and for the microenvironmental immune cell compartment (differentiation, activation, and cytokine secretion), that promotes tumor progression (**He W et al. 2016** and **Prinsloo E et al. 2012**). Therefore, in order to discover if these client proteins are involved in the growth and development of T-ALL and B-ALL cells, we treated our cells with drugs that inhibit AKT, such as MK-2206 or drugs that inhibit STAT3, such as Niclosamide. However the viability of T-ALL and B-ALL cells was not affected, and by western blot, we found that neither STAT3 nor AKT was phosphorylated in T-ALL and B-ALL cells. In this study, we found that HSP90 is overexpressed in ALL cells compared to resting B and T cells. HSP90 overexpression is accompanied by an overexpression of LYN kinase in B-ALL and LCK kinase in T-ALL. We also discovered that HSP90 is an important regulator of SRC kinases, which are involved in the intracellular signaling pathways necessary for the growth and proliferation of T-ALL, B-ALL and other types of leukemic cells. Through immunoprecipitation, we have demonstrated that these tyrosine kinases are present in a chaperone-client complex with HSP90. This explains the sensitivity of primary T-ALL and B-ALL samples to NVP-BEP800 (HSP90 inhibitor), which correlates with the expression of SFKs by these cells.

3. HSP90 Inhibitors

Numerous inhibitors of HSP90 have been discovered in the past 15 years (**Ho N et al. 2012; Lu X et al. 2012; Garcia-Carbonero R et al. 2013; Bhat R et al. 2014**). The majority of these developed HSP90 inhibitors inhibit HSP90 ATPase activity by binding to the N-terminal ATP-binding pocket and few of them target the C-terminal ATP binding site. For example, Geldanamycin (**Miyata Y. 2005**), 17-N-allylamino17-demethoxyGeldanamycin (known as 17AAG) (**Usmani S et al. 2009**), Ganetespib (also known as STA-9090), NVP-AUY922 and NVP-BEP800 (**Lin S et al. 2017** and **Stingl L et al. 2010**) target the ATP binding pocket at the N-terminal domain, while Cisplatin and Novobiocin were found to bind the C-terminal domain of HSP90 (**Donnelly A and Blagg B. 2008**). Although HSP90 inhibitors like 17AAG, 17 DMAG and NVP-AUY922 have shown promising therapeutic outcomes in clinical trials in various types of cancer, they have undesirable properties such as potential toxicity because they target the NTD for both HSP90 isoforms: HSP90 α and HSP90 β.

We were interested in the use of novel isoform-selective HSP90 inhibitor, NVP-BEP800, which specifically inhibits HSP90β by blocking its N-terminal ATP-binding pocket, and gives better clinical outcomes than pan-HSP90 inhibitors (**Yim K et al. 2016**). In addition, it enables studies to be performed on the roles of the different HSP90 isoforms. To confirm our selection, we tested the effect of NVP-BEP800, NVP-AUY922 and 17AAG on the viability of T-ALL and B-ALL cell lines by using XTT assay, and we found that NVP-BEP800 was more potent than the other HSP90 inhibitors.

The majority of experiments with NVP-BEP800 were performed *in vitro* not *in vivo*. These experiments, explored the impact of HSP90 inhibitors in the migration and invasion of lung carcinoma and glioblastoma (**Hartmann S et al. 2013**), and tested the effects of NVP-BEP800 on apoptosis and proliferation of multiple myeloma cells, fibrosarcoma and other cancer cell lines (**Stühmer T et al. 2009**). Also, a previous experiment showed that NVP-BEP800 with another HSP90 Inhibitor radiosensitise tumor cells through cell-cycle impairment and DNA damage (**Stingl L et al. 2010)**. In vitro analysis also indicated that HSP90β enhances the tumor vasculogenic mimicry (VM) in mice with hepatocellular carcinoma, and the HSP90 inhibitor NVP-BEP800 suppresses VM formation by targeting HSP90β (**Meng J et al. 2018**). In breast cancer models, NVP-BEP800 modulates HSP90 client proteins and downstream signaling pathways causing antitumor activity. Moreover, a study determined that the viral latency associated nuclear antigen (LANA) and the viral co-receptor EPHA2, which are essential for Kaposi Sarcoma-associated herpes virus (KSHV) infection, are client proteins of HSP90, and that NVP-BEP800 is efficacious against KS by downregulating the levels of these proteins (**Chen W et al. 2012**).

Herein, we examined the antiproliferative activity of NVP-BEP800 in a collection of B-cell acute lymphoid cell lines, such as Raji, Daudi and BALL-1, as well as in a collection of T-cell acute lymphoblastic leukemia cell lines, such as Jurkat and RPMI-8402. We showed that HSP90 inhibition caused a significant decrease in proliferation and viability of all (ALL) cells lines, and in a dose-dependent manner. However as we said before, we did not find a correlation between HSP90 expression levels and the response to therapy, whereas a correlation was found between SRC kinases expression levels and the magnitude of response to treatment.

The levels of expression of HSP90 isoforms (HSP90 α; HSP90 β) in cell lines were unchanged in the treated group and the control group. This means that NVP-BEP800 deactivates HSP90 without affecting its expression. In addition to the non-significant increase in the expression of HSP70 in Raji, it was not affected in Jurkat after treatment, nor was the level of BCL2 involved in apoptosis.

However, the level of cleaved Caspase-3 was increased confirming that treated ALL cells underwent apoptosis. This is shown in more detail later.

Our preclinical study on PDX models shows that NVP-BEP800 delays the progression of T-ALL and slows down the death of animals by about 14 days. On the other hand, it delays the development of B-ALL and slows down the death of mice by about 7 days.

In fact, I injected 10 mg/kg of the molecule three times, on day 20, 25 and 30 after T-ALL cell transplantation. While I injected 10 mg/kg of the molecule three times on day 15, 20 and 25 days after B-ALL cell transplantation. If we had optimized the treatment dose to 20 or 30 mg/kg, we would probably notice a better effect of the molecule. Regarding the toxicity of the molecule, as we mentioned above, NVP-BEP800 is an isoform selective inhibitor of HSP90, so it must have a favorable safety and efficacy profile. A previous in vivo study showed that NVP-BEP800 provided a high degree of flexibility in dose and schedule within a clinical setting, as mice in in vivo trials began to lose body weight when NVP-BEP800 was administered at a dose greater than 40mg/kg daily over a two-week period (**Massey A et al. 2010**). In our experiments, we confirmed the low toxicity of this drug in vivo, by administering three i.v injections of NVP-BEP800 at a rate of 10mg/kg every five days, and we showed that the drug had no negative effect neither on the development of mice's body weight nor on hematologic parameters. When peripheral blood was analyzed at day 15, using a hemocytometer, we found that the percentages of lymphocytes, monocytes, granulocytes, eosinophils were nearly the same in the group of mice treated with the drug and in the control group. We also found that the levels of hemoglobin and hematocrit and the counts of platelets, white blood cells and red blood cells were almost the same in both groups. Additionally, we found that drug showed no negative effect on the mice's vitality after monitoring the mice for more than 8 weeks.

4. Role of SRC kinases in T-ALL and B-ALL

The role of SRC kinases in acute lymphoblastic leukemia has been recently elucidated in murine models of leukemia (**Li S. 2007**). LCK is most commonly expressed in T cells, during T cell development; it plays a critical role in relaying pre-TCR- and TCR-mediated signaling (**Ballek O et al. 2017** and **Rossy J et al. 2012**). Many works identified and validated the SRC family kinase LCK as a protein whose activity is absolutely required for the proliferation and survival of T-cell acute lymphoblastic leukemia cells (**De Keersmaecker K et al. 2014** and **Bommhardt U et al.**

2019). The LCK pathway was found to be of interest and LCK was identified as a new potential drug target in T-ALL cells.

In addition, LYN is predominantly expressed in B-lymphocytes and plays a central role in initiating B-cell signaling. Evidence is mounting that strongly implicates an important role for LYN in several types of leukemia and lymphoma (**Donato N et al. 2003** and **Contri A et al. 2005**), particularly in B-cell acute lymphoblastic leukemia (**Hu Y et al.2004**). In B-cell lymphocytic leukemia, studies have confirmed the overexpression of LYN and its critical role in maintaining proliferation and anti-apoptotic pathways in leukemic cells (**Tibaldi E et al. 2011**). Therefore, LYN pathway was considered a pathway of interest and LYN was identified as a new potential drug target in B-ALL cells.

HSP90 has been shown to play a protective role in the regulation of SRC family proteins, as in neutrophils increasing cell survival (**Gupta S et al. 2018**), or in endothelial cells allowing regulation of the vascular endothelial growth factor receptor (**Meng J et al. 2017**). A multipotent complex containing HSP90 and LCK has been already described in T cells (**Lowenberg M et al. 2006**), HSP90 also has been found associated to LYN in B-chronic lymphocytic leukemia (**Trentin L et al. 2008**). After proving that LCK and LYN kinases are clients of HSP90, we showed that NVP-BEP800 affects the viability of T- and B-ALL cells by dysregulating these kinases involved in the proliferation of ALL cells, also we showed that NVP-BEP800 slows the progression of disease in xenograft leukemia models, when used in vivo.

Compared with normal hematopoietic progenitors, a high level of tyrosine phosphorylation was detected in most AML and ALL samples. The SRC family kinases appeared primarily activated in nearly all cases of leukemia. Among SFKs, LCK was consistently expressed at a high level and constitutively activated in T-ALL cells (**Serafin V. et al. 2017**) and LYN was consistently expressed at a high level and constitutively activated in B-ALL cells (**Dai H et al. 2020**). In this study, we provided strong evidence that T-ALL and B-ALL cells expressed relatively high amounts of LCK and LYN constitutively phosphorylated on the activation loop that are important for the proliferation and growth of ALL cells in PDX models, as well as in cell lines, and that the sensitivity of ALL cells to NVP-BEP800 was based on their level of expression for SRC but not for HSP90.

To confirm our evidence, we used two T-lymphoid cell lines, the Rpmi-8402 line not expressing LCK and the Jurkat line expressing LCK and we treated both cell lines with NVP-BEP800. By western blot, we found that the drug affects the level of phosphorylated LCK and the total amount of LCK in the Jurkat line, while no change was detected regarding the level of LCK in Rpmi-8402

line. Both cell lines were expressing HSP90 even after treatment. Also, the viability test showed that Jurkat cells were more sensitive to the drug, compared to Rpmi-8402 cells, proving that the sensitivity of T-ALL cells to NVP-BEP800 was dependent on their level of expression for LCK and p-LCK. On the other hand, we used four B-lymphoid cell lines (Raji, Daudi, REH and BALL-1), by western blot we found that each cell line had a different level of phosphorylated LYN and a different total amount of LYN than other lines, and we found that the levels of proteins decreased after treatment with NVP-BEP800 in all cell lines. The viability test also showed that the sensitivity of these cell lines was correlated with the quantity of LYN and p-LYN measured by western blot, regardless of the HSP90 level.

Several studies verified that SRC kinases are critical factors for maintenance of T-ALL and B-ALL cells in vitro and in vivo, by downregulating the expression of theses kinases. They showed that knock down of LCK and other SRC kinases leads to proliferation reduction of ALL cells. They also showed that knockdown of SRC kinases in dexamethasone-resistant ALL cells lead to resensitisation of these cells to dexamethasone (**Shi Y et al.2020**). In our study, we knocked down the expression of LCK and LYN using lentivirus-expressing shRNA. We showed that with the decrease in the expression of SRC kinases, ALL cells undergo cell cycle arrest and apoptosis in addition to the loss of sensitivity to NVP-BEP800.

Furthermore, studies have shown a positive correlation between LCK or LYN activation and the sensitivity of leukemic cells to Dasatinib (**Shi Y et al.2020** and **Kim YJ et al. 2016**). In our primary T-ALL and B-ALL cells, we found a correlation between the levels of expression of SRC and the sensitivity to NVP-BEP800, however, no correlation was found between the HSP90 expression levels and the sensitivity to drug.

In T-ALL, LCK can control the activation of RAS/MAPK, AKT/mTOR and the calcineurin/NFAT signaling which leads to the dephosphorylation of nuclear factor of activated T cells 1 (NFAT1) and its activation. NFAT1 is found essential for T-ALL cells' function and survival (**Serafin V et al. 2017**). Treatment with NVP-BEP800 removed LCK phosphorylation and deactivated the calcium/NFAT pathway. NFAT1 became phosphorylated when inactivated and leaves the nucleus, inhibiting T-ALL cell survival, growth and maintenance.

In B-ALL, LYN along with other tyrosine kinases plays a crucial role in the BCR-induced activation of NFκB which is required for survival of activated leukemia cells (**Davis R et al. 2001** and **Ma C et al. 2020**). However, after treatment with NVP-BEP800 the entire BCR signaling pathway was

deregulated, LYN and NFκB lost their phosphorylation and became inactive, the latter leaves the nucleus which leads to inhibition of survival, growth and maintenance of B-ALL cells.

The results above were confirmed by analyzing the transcription of some genes involved in the cell cycle and apoptosis. The balance between the pro- and anti-apoptotic proteins was deregulated after treatment. The drug has increased the transcription of pro-apoptotic genes like (*BCL2L1, BAD, BAX and BIM),* as well, it has decreased the transcription of anti-apoptotic genes like (*CDKN1A*) (**Ola M et al. 2011**). Furthermore, by Annexin V staining and flow cytometry we confirmed that NVP-BEP800 treatment arrested these ALL cells in the G0/G1 phases of the cell cycle due to repression of *Cyclin D3* and *c-Myc*, which are both involved in the cell division cycle (**Ausserlechner M et al. 2004**). In addition, we confirmed that leukemic cells underwent apoptosis after treatment by cytometry that showed an increase in the percentage of cells undergoing apoptosis, and by western blot that showed an increased level of cleaved Caspase-3.

5. NVP-BEP800 effect on active kinome

Studies have shown that in breast cancer cells and in melanoma, NVP-BEP800 induces client proteins degradation (including ERB-B2, B-RAFV600E, RAF-1, and AKT) leading to potent antitumor activity in breast cancer xenograft models and melanoma cells (**Massey A et al. 2010** and **Mielczarek-Lewandowska A et al. 2020**). Meng J et al showed that NVP-BEP800 reduces VEGFR1 and VEGFR2 expression and inhibits hepatocellular carcinoma growth and tumor angiogenesis (**Meng J et al.2017**). Our work indicates that NVP-BEP800 leads to a decrease in cell viability of ALL cells, by reducing LCK and LYN activity, which leads to cell cycle arrest and apoptosis. We found that the AKT pathway was unaffected, because AKT was not phosphorylated in our T-ALL and B-ALL models and was not involved in the growth and progression of leukemic cells. STAT3 also, was not phosphorylated and was not involved in the development of leukemia in our PDX models. Moreover, drugs that inhibit AKT such as MK-2206, and drugs that inhibit STAT3 such as Niclosamide, did not affect the viability of T-ALL and B-ALL cells.

Since BEP800 has an inhibitory effect on the active kinome which go beyond its inhibitory effect on the SRC family, it would therefore be interesting to characterize the effect of NVP-BEP800 on other pathways that could be involved in leukemia progression, like EGFR-RAS and RAF-MEK-ERK pathways, in order to better understand the mechanism of action of this molecule in acute lymphoblastic leukemia.

6. The role of SRC kinases in cancer treatment resistance

Resistance to chemotherapy is believed to be a major cause of treatment failure in cancer. Several studies have talked about a specific role of SRC kinases in the acquired resistance of cancer cells to chemotherapy, and that inhibiting these kinases overcome the resistance of cancer cells to chemotherapy (**Formisano L et al. 2014**).

Ischenko I et al found that SRC specifically regulates 5-FU (5-fluorouracil) chemosensitivity in chemotherapy-resistant cell lines and that the inhibition of SRC by Saracatinib augment the chemosensitivity of (5-FU)-resistant human pancreatic cancer cells to 5-FU (**Ischenko I et al. 2008**). Another study showed that LYN was upregulated in Estrogen receptor–positive ER+ breast cancer lines resistant to estrogen receptor antagonists, and that the inhibition of SRC kinase (LYN) enhances the antitumor effect of ER antagonists against the resistant ER+ breast cancer lines (**Schwarz L et al. 2014**).Also , it has been showed that the overactivation of LCK, promoted the resistance of ovarian cancer cells to Cisplatin (chemotherapy drug), and that the inhibition of LCK would increase the Cisplatin efficacy (**Crean-Tate K et al. 2021**).

7. The role of SRC kinases in ALL treatment resistance

Several studies concerning various hematologic malignancies have revealed the role of SRC kinases in resistance to glucocorticoids (GC), proteasome inhibitors and other chemotherapies, especially in lymphocytic leukemia and lymphoma models, and that their inhibition markedly induces glucocorticoids and proteasome inhibitors (PIs) sensitivity (**Harr M et al. 2010** and **Kim A et al. 2015**).

Wei et al found that Rapamycin induces GC sensitivity in ALL by inhibiting MCL1, the downstream target of the PI3K/Akt/mTOR pathway (**Wei G et al. 2006**). Other kinases involved in GC resistance in infant ALL might be the SRC kinases, particularly LCK. Interestingly, SRC kinases bind to HSP90, the chaperone of the GR complex. Upon stimulation of glucocorticoid, GR is not only released from its co-chaperones, but the associated SRC kinases also separate from the GR-complex. Inhibition of SRC kinase by selective SRC kinase inhibitors, overcomes GC resistance in lymphoma cells (**Harr M et al. 2010**). Furthermore, studies have shown that glucocorticoid resistance resulted from the activation of the calcineurin / NFAT pathway and IL- 4 production regulated by LCK hyperactivation (**Serafin V et al. 2017**). They demonstrated that the

downregulation of SRC kinases by SRC kinase inhibitors and siRNA induced GC sensitivity of in vivo and in vitro prednisolone-resistant ALL cells (**Spijkers-Hagelstein J et al. 2013**).

Kim et al demonstrated that proteasome inhibitors-resistant Lymphoma cells showed a high increase in the expression of the B-cell receptor (BCR) components. Activation of the BCR signaling pathway enhanced the activity of SRC family kinases (SFKs), especially LYN, and downstream kinases PI3K/AKT/mTOR in PI-resistant Lymphoma cells. It has been shown that depletion of LYN significantly reduces several kinase activities, preventing lymphoma cells proliferation (**Kim A et al. 2015**). In addition, Okabe et al demonstrated that activation of LYN plays a crucial role in the survival of leukemic cells resistant to Nilotinib (BCR/ABL inhibitor). They found that the inhibition of LYN kinase activity by Dasatinib (SRC kinase inhibitor) induces the apoptosis of Nilotinib-resistant leukemic cells (**Okabe S et al. 2011**).

Thus, based on the studies mentioned above, inhibiting LCK and LYN represents a solution to improve the effect of glucocorticoids and other chemotherapies in resistant cancer cells. Here comes the importance of combining our drug (NVP BEP800) with antineoplastic drugs to treat resistant leukemic cells and aggressive tumors.

8. Use of NVP-BEP800 in combination with other treatments

NVP-BEP800 has an effect on the viability of ALL cell lines and on PDX mice developing ALL, as it prolonged the survival of mice by interfering with leukemia progression.

Nevertheless, this molecule was not found to be sufficient to definitely prevent leukemia expansion in our xenograft models. Despite the NVP-BEP800 treatment, however, the surviving cells continued to invade the bone marrow and spleen of animals probably due to the activation of other pathways involved in proliferation of leukemic cells.

Since preclinical and early-phase clinical studies indicate improved antitumor activity when HSP90 inhibitors are combined with chemotherapies or targeted agents, it would suit therefore to use multiple molecules in combination to simultaneously target several pathways. The table **(Table 9**) below summarizes the additive or synergistic effects of HSP90 inhibitors and anti-cancer drugs observed in most cases.

HSP90 inhibitors	Drugs in combination	Interaction	Cancer cell type	References
GA, Radicicol	Cisplatin, Oxaliplatin	*synergistic*	Colon, Glioma, solid tumors	Moser C et al. 2007 ; Roe S et al. 1999 ;Rosenhagen M et al. 2003
GA, Radicicol	Topoisomerase II poison (Etoposide) (VP16),	*synergistic*	colon (HCT 116)	Barker C. 2006 ; Yao K et al. 2007
17-AAG	Bortezomib (PS-341, proteasome inhibitor)	*synergistic*	leukemia, multiple myeloma, breast (MCF-7 cell)	Mitsiades C. 2005 ; Mitsiades C et al. 2003; Mimnaugh E et al. 2004
17-AAG	UCN-01 (7hydroxystaurosporine) , chk1 inhibitor	*synergistic*	U937 monocytic leukemia cells	Jia W et al.2003
17-AAG	Imatinib mesylate/PD1 80970	*enhancement*	leukemia (CML)	Radujkovic A et al. 2005 ; Gilbert J et al. 2010
17-AAG	PKC412 (FLT3 tyrosine kinase inhibitor)	*synergistic*	AML (cell culture)	George P et al. 2004
17-AAG	GTP14564(FLT3 tyrosine kinase inhibitor)	*synergistic*	leukemia(leukemias with FLT3 mutations)	Yao Q et al. 2005
NVP-AUY922	Doxorubicin	*synergistic*	MCF-7 breast cancer	Mohammadian M et al. 2020
NVP-AUY922	inhibitor of PI3K/mTOR(PI-103)	*synergistic*	Glioblastoma cell lines	Gaspar N et al. 2010

Table 9: Synergistic effects of anti-cancer drugs and HSP90 inhibitors

Although several studies that supported the hypothesis of synergy provided by combining HSP90 inhibitors with TKIs to reduce cancer progression pathways (**Workman P et al. 2007** and **Whitesell L et al. 2014**), however, in our study, we found that the combination of NVP-BEP800 with Dasatinib, a tyrosine kinase inhibitor did not improve the inhibitory effect of the HSP90 inhibitor alone, on the viability of B-ALL and T-ALL cells. This is probably because the NVP-BEP800, by itself, showed a pertinent dysregulation of LCK phosphorylation in T-ALL and LYN phosphorylation in B-ALL. Herein, we showed that NVP-BEP800 affected not only the phosphorylated forms of SRC but also the total amount of SRC kinases in cells.

Concerning our drug, only a few works explored the impact of NVP-BEP800 in combination with other therapies on the proliferation and invasion of cancer cells (**Hartmann S et al. 2013**). The majority of these studies suggest that ionizing radiation (IR) enhances the inhibitory effect of NVP BEP800 , by destabilizing several HSP90 client proteins causing S-phase depletion and G2 / M interruption, increased DNA damage ,prolonged repair, and to some extent apoptosis of lung carcinoma and glioblastoma cells (**Wu J et al. 2014** and **Stingl L et al. 2010**).

Our experience with combination therapy of HSP90 inhibitors and TKIs should not disappoint us or prevent us from searching for novel potential combination therapies to treat acute lymphoblastic leukemia. Combined NVP-BEP800-IR treatment and co-treatments of this HSP90 inhibitor with monoclonal antibodies like Blinatumomab (**Guerra V et al. 2019**) or with chemotherapy drugs like Doxorubicin and Vincristine seems promising and could lead to a prospective approach in treating Acute Lymphoblastic Leukemia by incorporating these agents into treatment regimens.

Conclusion and future perspectives

In this thesis my goal was first to elucidate the expression and the functional roles of HSP90 and SRC tyrosine kinases in T-cell and B-cell acute lymphoblastic leukemia. Our principal findings showed that LCK is highly expressed in T-ALL, while LYN is highly expressed in B-ALL. Also we found that HSP90 is more expressed in leukemic cells than in normal hematopoietic cells.

HSP90 and SRC kinases have been shown to have important role in growth, survival and proliferation of cancer cells, especially leukemic cells.

Our findings have confirmed the chaperone-client interactions between HSP90 and SRC kinases in ALL cells. Moreover, we confirmed the correlation between the expression of the SRC kinases in ALL cells and their sensitivity to the NVP-BEP800. Thus based on what we emphasized above, we proved that targeting HSP90, by NVP-BEP800, is a novel and promising therapeutic approach for

the treatment of ALL, as it targets LCK and LYN signaling pathways important for leukemia progression (**Figure 17)**.

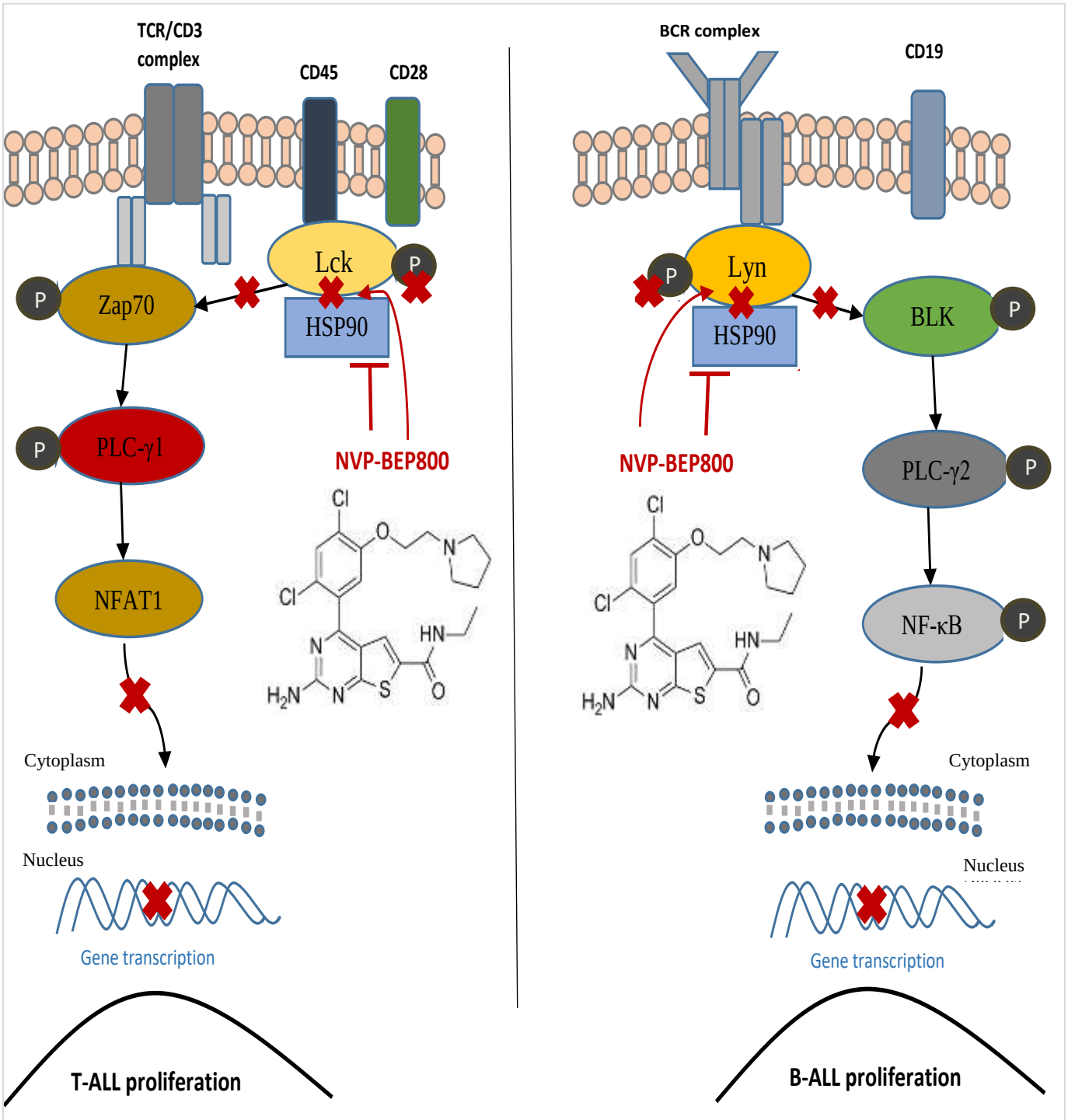


Figure 17: The potential targets of HSP90 inhibitors in T-cell and B-cell ALL. Schematic representation of LCK and LYN signaling pathways and the potential targets for therapy with HSP90 inhibitors in T-cell and B-cell acute lymphoblastic leukemia.

HSP90 has a growing list of client proteins, and in this particular study, LCK and LYN were confirmed as client proteins of HSP90. However, more works need to be done to identify the members of pathways that could be the reason behind the resistance of surviving leukemic cells. The identified proteins should then be analyzed and studied to discover their potential role in the treatment of acute lymphoblastic leukemia. Works also needs to be done to elucidate the cellular mechanism of action of NVP-BEP800 in inducing leukemic cell death by measuring cellular Ca+2 homeostasis, and the kinases which are involved in cell death.

HSP90 inhibitors have been shown to exhibit synergistic activity when used in combination with other anti-cancer agents (**Mitsiades C et al. 2006**). The combination of HSP90 inhibitor (17AAG) with Bortezomib showed a therapeutic effect in patients with leukemia and multiple myeloma (**Mimnaugh E et al. 2004**), another combination of HSP90 inhibitor (GA) with Cisplatin has showed an important effect in solid tumors (**Solar P et al. 2007**). Moreover, NVP-AUY922 showed synergistic effect in breast cancer and glioblastoma when used in combination with Doxorubicin or with PI-103 (inhibitor of PI3K/mTOR) (**Mohammadian M et al. 2020** and **Gaspar N et al. 2010**). Future trials tailored to specific clients in acute lymphoblastic leukemia are needed to determine the potentially beneficial combination of NVP-BEP800 and other chemotherapy drugs.

My dissertation demonstrated that the chaperoning of SRC family kinases by HSP90 is involved in the growth and proliferation of ALL. These novel findings provide an alternative approach to target SRC kinases by HSP90 inhibitors, and make it possible to consider a new therapeutic strategy to reduce the intensity and toxicity of existing therapies and to treat patients with relapsed acute lymphoblastic leukemia.

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V.ANNEX

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High-fat diet intensifies *MLL-AF9*-induced acute myeloid leukemia through activation of the FLT3 signaling in mouse primitive hematopoietic cells

François Hermetet[1,2,4], Rony Mshaik[1,4], John Simonet[1], Patrick Callier[3], Laurent Delva[1,2] & Ronan Quéré[1,2]✉

Using a *MLL-AF9 knock-in* mouse model, we discovered that consumption of a high-fat diet (HFD) accelerates the risk of developing acute myeloid leukemia (AML). This regimen increases the clusterization of FLT3 within lipid rafts on the cell surface of primitive hematopoietic cells, which overactivates this receptor as well as the downstream JAK/STAT signaling known to enhance the transformation of *MLL-AF9 knock-in* cells. Treatment of mice on a HFD with Quizartinib, a potent inhibitor of FLT3 phosphorylation, inhibits the JAK3/STAT3, signaling and finally antagonizes the accelerated development of AML that occurred following the HFD regimen. We can therefore conclude that, on a mouse model of AML, a HFD enforces the FLT3 signaling pathway on primitive hematopoietic cells and, in turn, improves the oncogenic transformation of *MLL-AF9 knock-in* cells and the leukemia initiation.

The obesity-related cancer burden represents up to 9% of all cancer cases[1]. Even if major role has been established for high-fat diet (HFD) in several solid cancers (e.g. gastric cardia, colon, rectum and liver cancer)[2], only a small number of epidemiological studies have been conducted on leukemia. A study provides the evidence for an association between a Western dietary pattern and chronic lymphocytic leukemia, suggesting that a proportion of cases could be prevented for this disease by modifying dietary habits[3]. Among hematological diseases, diet-induced obesity has been shown related to myeloma development[4]. Although acute myeloid leukemia (AML) is a relatively rare disease, accounting for roughly 1.2% of cancer deaths, its incidence should increase as the population ages. In a cohort of more than half a million individuals, those eating large quantities of food were more likely to develop AML[5]. In addition, a higher body mass index is associated with poorer survival in pediatric AML[6]. The leukemia burden of AML is also much higher in HFD-induced obese mice[7,8].

We and others have already shown that HFD induces major perturbations in murine hematopoietic cells, such as a loss of the hematopoietic stem cells (HSC), as well as in the homeostasis of the mouse hematopoietic system[9–15]. The role of HFD on the promotion of leukemia is however still poorly explained, the data available are sparse concerning HFD impact on disease initiation and development in AML, and there is no study, to the best of our knowledge, on the HFD potential mechanism of action leading to the transformation of normal primitive hematopoietic cells into AML cells.

In this study, we therefore investigated whether a HFD could accelerate the risk of developing AML. The *MLL-AF9 knock-in* mouse model reflects aspects of AML in humans and it can be used to provide biological insights into MLL-rearranged leukemogenesis[16,17]. The purpose of this study was first to describe how feeding mice a HFD over a short period influences the initiation and development of AML and then to characterize how the consumption of a HFD transforms primitive hematopoietic cells.

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Materials and methods

Use of experimental animals. The ethics committee for animal welfare of the University and the French ministry of higher education and research approved all animal experiments (under reference APAFIS#16187-2018071914379464v3). We confirm that all experiments were performed in accordance with relevant guidelines and regulations of this committee.

Mice. *MLL-AF9* (Kmt2a<tm2(MLLT3)Thr>/KsyJ, Jackson Laboratory), C57bl/6J (Charles River and Envigo) and C57bl/6.SJL (Ly.1) mice (Charles River) were kept in the animal facility at the University of Burgundy. Genotyping of *MLL-AF9 knock-in* mice was done following a protocol described by the manufacturer online (https://www.jax.org/strain/009079). Mice were maintained on a rodent chow diet ad libitum, composed of 4% fat for the control diet (CD) or 42% fat for the high-fat diet (HFD; MD. 88137, Envigo, France). Quizartinib (SelleckChem) was reconstituted at 10 mg/mL in Dimethyl Sulfoxyde (DMSO) and injected in the peritoneum at 5 mg/kg, twice per week, during the 4 weeks of HFD feeding.

Transplantations. The BM cells (2×10^5 cells) isolated from *MLL-AF9 knock-in* mice were transplanted into the tail veins of lethally irradiated (900 cGy, Biomep, Dijon, France) C57bl/6J recipient mice. Thirty days after transplantation, the mice were divided into two groups: one was fed a HFD for 4 weeks and the other was fed a CD. After 4 weeks of a HFD, the mice were fed a CD ad libitum. For the cell-transforming potential (CTP) study, mice were transplanted with 2×10^3, 2×10^4 or 2×10^5 cells isolated from *MLL-AF9 knock-in* mice together with 2×10^5 Ly.1 supported cells isolated from the BM of a wild-type C57bl/6J mouse. We analyzed AML development in lethally irradiated recipient Ly.1 mice, by transplantation of 2×10^4 AML cells with 2×10^5 Ly.1 supporter cells.

Peripheral blood (PB) and bone marrow (BM) cells analyses. After tail vein PB sampling, white blood cells (WBC) were counted with a hemocytometer (SCIL Vet ABC$^+$, Oostelbeers, The Netherlands). PB was monitored monthly for occurrence of AML. Bones were crushed in a mortar and total BM cells were filtered (30 μm).

Flow cytometry and FACS. For staining of HSC and progenitors, we used antibodies and strategies for gating as previously described[10]. For western blot and immunostaining, c-KIT$^+$ cells were isolated from BM after 4 weeks of CD or HFD with magnetic murine CD117-Microbeads (Miltenyi Biotec). Magnetically lineage-depleted (Lin$^-$) BM cells (Miltenyi Biotec) were stained in PBS 1x with combinations of antibodies. We used CD45.2-PE-Cy7, c-KIT-Pacific Blue (PB), SCA1-APC-Cy7, FLT3 (CD135)-PE, CD34-AF647, CD16/32-FITC and IL7Rα-PE-CF594 on Lin$^-$ cells, 4-weeks after the CD or HFD. To analyze phenotype of AML, MAC1-AF647 and GR1-FITC (BD Biosciences) were used on total BM cells, when mice have developed leukemia. We used KI67-FITC (BD Biosciences) and propidium iodide (BD Biosciences) on AML cells isolated ex vivo from BM and permeabilized for intracellular staining (BD Cytofix/Cytoperm, BD Pharmingen). Cell subsets were analyzed using a FACS Canto10 or a LSR-Fortessa (BD Biosciences) and sorted on a FACSAriaIII cell sorter (BD Biosciences). Data were analyzed using FlowJo software (version 10, TreeStar Inc, https://www.flowjo.com).

Western blot. Western blot was performed on c-KIT$^+$ cells isolated with magnetic murine CD117-Microbeads (130-091-224, Miltenyi Biotec) from BM after 4 weeks of CD or HFD. We used antibodies to detect FLT3 (PA5-34448, Thermo Fisher Scientific) or ACTB (612656, BD Biosciences). We used JAK3 (#8863), phospho-JAK3 (Y980/981) (#5031), STAT5 (#94205), phospho-STAT5 (Y694) (#9359), STAT3 (#4904), phospho-STAT3 (Y705) (#9145) antibodies, all from Cell Signaling. After immunoprecipitation of all tyrosine phosphorylated proteins with an anti-phosphotyrosine antibody (05-321, Millipore), FLT3 was analyzed by western blot (PA5-34448, Thermo Fisher Scientific). After immunoprecipitation with an anti-FLT3 antibody (PA5-34448, Thermo Fisher Scientific), phosphorylation of FLT3 was assessed with an anti-phosphotyrosine antibody (05-321, Millipore). Gel images were processed and analyzed for quantification (Fiji, NIH).

Immunofluorescence and microscopy. After 4 weeks of the CD or HFD, c-KIT$^+$ cells were isolated with magnetic CD117-Microbeads (Miltenyi Biotec) from BM. Cells were stained with rabbit anti-FLT3 (PA5-34448, Thermo Fisher scientific) and secondary anti-rabbit-AF488 antibodies (Thermo Fisher Scientific). Lipid rafts were stained with cholera toxin subunit B conjugated with AF555 (Thermo Fisher Scientific). Cells were placed on glass slides for 5 min. ProLong Gold Antifade Mountant containing DAPI (Thermo Fisher Scientific) was applied directly to fluorescently labeled cells on microscope slides. Fluorescence was observed by microscopy (Axio Imager 2, Zeiss) and the images were processed (Fiji, NIH).

Reverse transcription quantitative polymerase chain (RTqPCR) reaction. After mRNA isolation with the RNeasy kit (Qiagen), M-MLV reverse transcriptase (Promega) was used to synthesize cDNA. The following TaqMan assays were then used for qPCR: *Flt3* (Mm00439016) and *Hprt1* (Mm03024075) used as endogenous controls. We used GoTaq Probe qPCR Master Mix (Promega). Experiments were carried out using the Viia7 system (Applied Biosystems).

DNA sequencing. Genomic DNA from AML cells isolated from the BM of 8 mice (from CD and HFD groups) that developed leukemia was extracted after cell lysis and protein precipitation (Qiagen); DNA was then precipitated with ethanol. PCR was performed with the following primers; *Flt3-e14-15-F*: TGCGACCATTGG GCTCTGTCTCCCCTTC; *Flt3-e14-15-R*: ACTGGC-CCTGACAGTGTGCATGCCCCC; *Flt3-e20-F*: GAGGAG

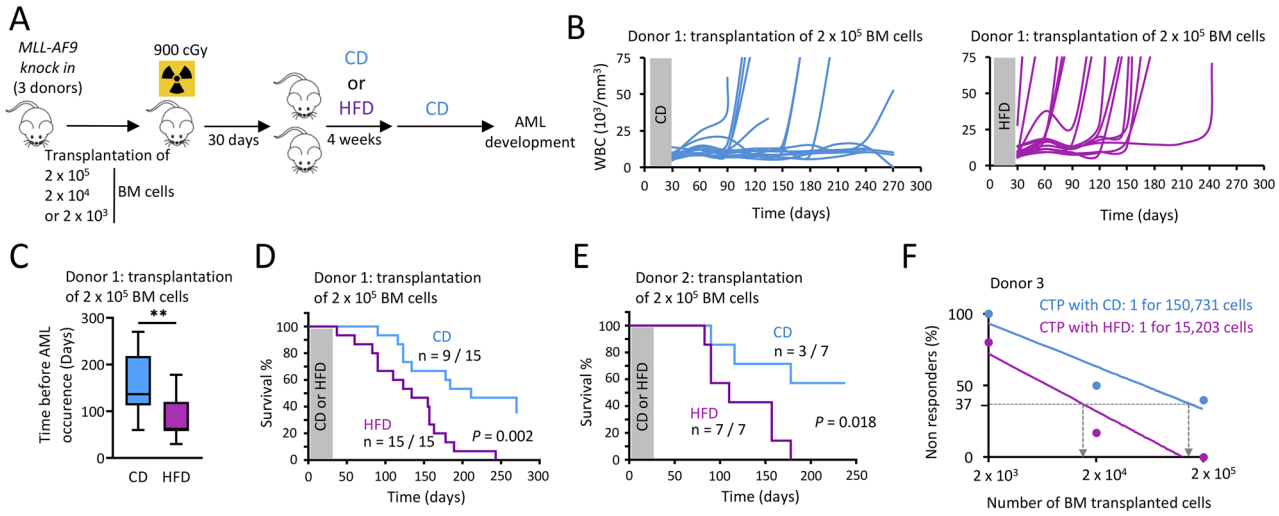

Figure 1. HFD increases the risk of AML development in mice. (**A**) Experimental workflow describing the procedure. Image performed with the GIMP software (version 2.10.18, GIMP, https://www.gimp.org/news/2020/02/24/gimp-2-10-18-released/). (**B**) Level of white blood cells (WBC) over time in the peripheral blood (PB) of mice fed with either a CD or a HFD. Mice fed a HFD developed more AML than CD-fed mice. PB was monitored monthly for occurrence of AML, n = 15 mice per group. (**C**) Time before observing AML occurrence following the BM cell transplantation, showing that this period is reduced for HFD-fed mice compared to CD-fed mice. We considered mice start to develop AML, when the WBC is over $15 \times 10^3/mm^3$ in PB. Data are presented as median (central line), first and third quartiles (bottom and top of boxes, respectively), and whiskers (extreme values); n = 9 and 15 mice for CD and HFD-groups, respectively. $^{**}P < 0.01$, two-tailed unpaired Student's t-test. (**D**) Survival curves showing that mice fed a HFD developed more AML than CD-fed mice, n = 15 mice per group; *P* value measured by Mantel–Haenszel test. (**E**) Survival curves with another *MLL-AF9 knock in* donor, n = 7 mice per group; *P* value measured by Mantel–Haenszel test. (**F**) Transplantation of varying numbers of *MLL-AF9 knock in* donor cells showing that the number of cell-transforming potential (CTP) increased when mice were fed a HFD. Transplantation of 2×10^3 cells, n = 5 mice; 2×10^4 cells, n = 5 mice; 2×10^5 cells, n = 5 mice.

GAAGATTTGAA-CGTGCTGACG; *Flt3-e20-R*: CCAGAGAAGGATGCCGTAGGACCAGACG. Sequencing was performed by Sanger (Genewiz).

CGH array. For the Array Comparative Genomic Hybridization (CGH array), we used the SurePrint G3 Mouse CGH Microarray Kit, 4 × 180 K (Agilent Technologies) and 1 µg of genomic DNA extracted from AML samples using Gentra Puregene Tissue kit (Qiagen). For analysis, we used the G2505 DNA microarray Scanner (Agilent Technologies) and the Agilent Cytogenomics software was used (version 2.7, Agilent Technologies, https://www.agilent.com/en/download-agilent-cytogenomics-software).

Statistics. All data were expressed as means ± SD or presented as median, first and third quartiles, and whiskers. Differences between groups were assessed with the Student's unpaired t-test. Statistical analysis of survival curves were assessed using the Mantel–Haenszel Logrank test. Statistics were performed using Prism 6 (GraphPad), significanceare indicated on the figures with the following convention: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Ethical standards. The ethics committee for animal welfare of the University and the French ministry of higher education and research approved all animal experiments (under reference APAFIS#16,187-2018071914379464v3). We confirm that all experiments were performed in accordance with relevant guidelines and regulations of this committee.

Results

HFD increases the risk of AML development in mice. We transplanted bone marrow (BM) cells from a *MLL-AF9 knock-in* mouse (Ly.2) into lethally irradiated C57bl/6.SJL (Ly.1) recipient mice that were divided in two groups: over a period of 4 weeks, one group was fed a control diet (CD) and the other a HFD (Fig. 1A). The HFD did not alter engraftment of *MLL-AF9 knock-in* Ly.2 BM cells to the BM, or the reconstitution of total white blood cells (WBC) in the peripheral blood (PB) of recipient mice (Supplementary Fig. S1). As we previously described in mice fed a 4-week HFD[10], among lineage negative (Lin^-) cells, we observed a decrease of the primitive HSC population ($SCA1^+$ c-KIT^+ $CD34^-$), but there was no impact on the distribution of other mature progenitors such as mega-erythroid progenitor (MEP), common myeloid progenitor (CMP), granulocyte/macrophage progenitor (GMP), and common lymphoid progenitor (CLP) (Supplementary Fig. S2).

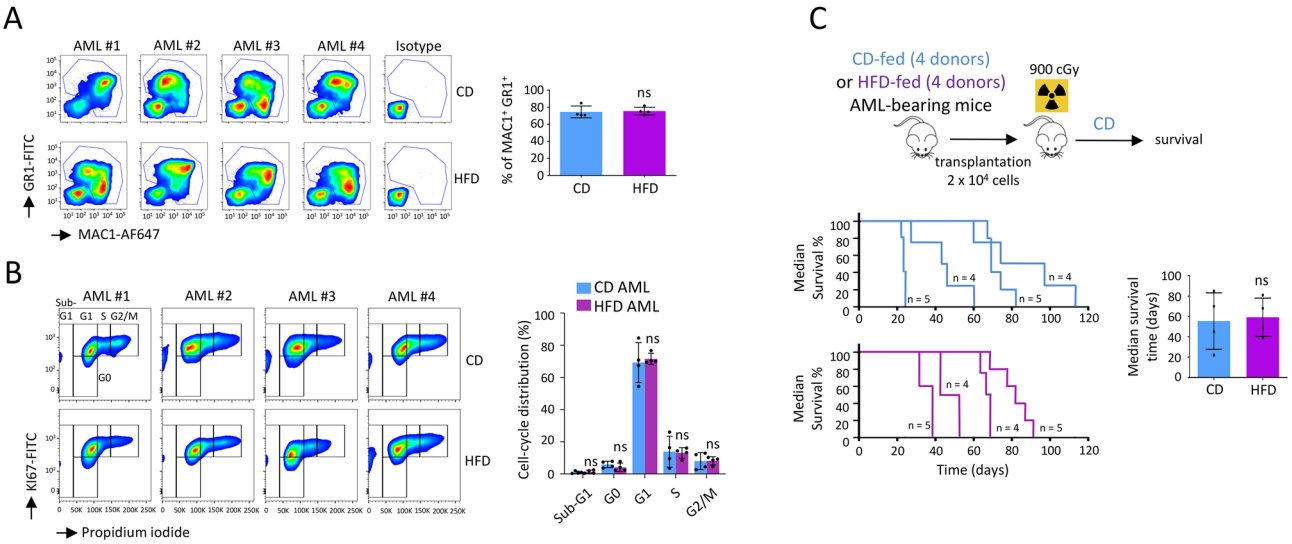

Figure 2. HFD did not modify the phenotype of resulting transformed AML cells. (**A**) AML cells isolated from BM expressed MAC1 and GR1 myeloid markers, with no difference observed between CD- and HFD-fed mice. Flow cytometry showing expression of MAC1 and GR1 and statistic of the percentage of leukemic cells in BM. Data show mean ± SD; n = 4 mice (#1–4) per diet group; *P* value measured by two-tailed unpaired Student's t-test; ns, non-significant. Data were analyzed using FlowJo software (version 10, TreeStar Inc, https://www.flowjo.com). (**B**) Cell cycle study performed by flow cytometry on AML cells isolated from BM of CD- and HFD-fed mice. Data show mean ± SD; n = 4 mice (#1–4) per diet group; *P* value measured by two-tailed unpaired Student's t-test; ns, non-significant. Data were analyzed using FlowJo software (version 10, TreeStar Inc, https://www.flowjo.com). (**C**) Post-CD and -HFD AML cells are similarly aggressive in vivo. Experimental workflow describing the procedure. Image performed with the GIMP software (version 2.10.18, GIMP, https://www.gimp.org/news/2020/02/24/gimp-2-10-18-released/). Survival curves after the transplantation of 2×10^4 AML cells isolated from 4 different AML-bearing mice from the CD and HFD groups (n = 4 samples analyzed in each group). Recipient mice (n = 4 or 5 transplanted mice with each samples of initial post-CD and -HFD AML cells) were fed a CD and monitored for occurrence of AML. Median survival time was calculated, n = 4 AML per diet group; *P* value measured by two-tailed unpaired Student's t-test; ns, non-significant.

HFD-fed mice developed AML much more quickly than did mice from the control group, as assessed by the increase in the total WBC count in the PB for HFD-fed mice (Fig. 1B), and times to AML occurrence ($P = 0.006$, Fig. 1C) and to death following AML development ($P = 0.002$, Fig. 1D). Using BM cells from another *MLL-AF9 knock-in* mouse donor, we confirmed that HFD accelerates development of AML ($P = 0.018$, Fig. 1E). Furthermore, after we transplanted different numbers of *MLL-AF9 knock-in* BM cells into lethally irradiated recipient mice, we determined that the risk for primitive hematopoietic stem/progenitor cells (HSPC) of being transformed into leukemia stem cells, mentioned as cell-transforming potential (CTP), was tenfold higher when mice consumed a HFD (Fig. 1F).

We can therefore conclude that HFD has intensified *MLL-AF9*-induced AML.

HFD did not modify the phenotype of resulting transformed AML cells. AML cells expressed MAC1 and GR1 myeloid markers, with no difference observed between CD- and HFD-fed mice (Fig. 2A). AML cells extracted ex vivo from BM were also active, as assessed by equivalent cell cycling activities measured by flow cytometry, after KI67 and propidium iodide staining (Fig. 2B). CGH-array showed no genetic alteration in DNA isolated from post-HFD AML cells, compared with post-CD AML samples (Supplementary Fig. S3). To check if the HFD induced leukemia with different levels of aggressiveness, we transplanted AML cells from primary mice into lethally irradiated C57bl/6.SJL (Ly.1) recipient CD-fed mice and then monitored for occurrence of AML. In these host mice transplanted with 2×10^4 AML cells from both CD and HFD-fed mice, we observed variability between AML samples to generate leukemia, without any significant difference between diet groups. Indeed, this experiment suggested that there is no variability in leukemia initiating cell (LIC) frequency between post-CD and -HFD AML samples (Fig. 2C).

We can therefore conclude that a HFD did not modify the phenotype of the resulting AML cells, their cell cycling activities, or their capacities to engraft and generate leukemia in recipient mice fed a CD.

HFD activates the FLT3/JAK3/STAT3 signaling on primitive murine hematopoietic stem/progenitor cells. Next, in order to elucidate the accelerated development of AML, we analyzed how a HFD affects signaling pathways on primitive HSPC isolated from BM. When we performed intracellular signaling by western blotting on primitive c-KIT$^+$ cells collected from mice after 4 weeks of HFD, we discovered an increased activation of the JAK-STAT pathway, as characterized by an improved phosphorylation of JAK3 ($P < 0.001$) and

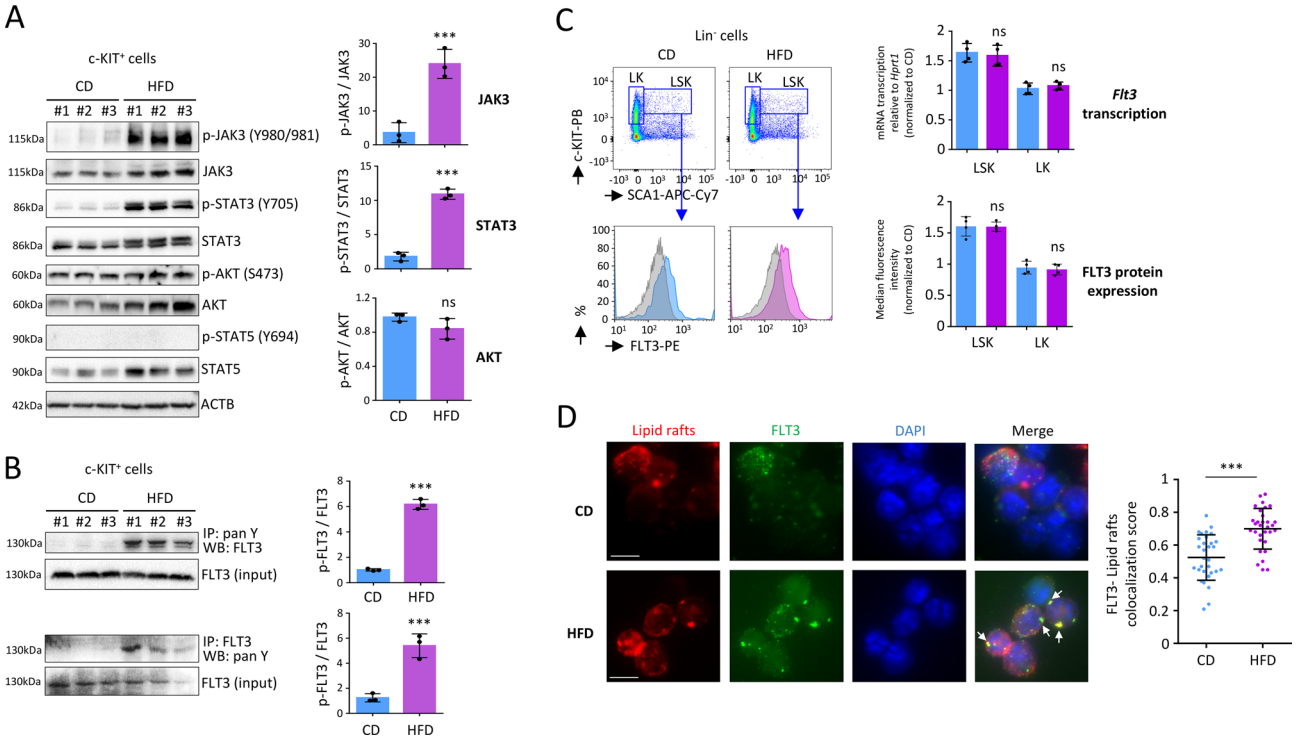

Figure 3. HFD activates FLT3/JAK3/STAT3 signaling on c-KIT⁺ BM cells after 4 weeks. (**A**) Western blot showing increased phosphorylation of JAK3 (Y980/981) and STAT3 (Y705) among the c-KIT⁺ *MLL-AF9 knock in* cells in BM, after 4 weeks of HFD. Data show mean ± SD; n = 3 mice per diet group; ***$P < 0.001$, two-tailed unpaired Student's t-test; ns, non-significant. Grouping of blots cropped from different gels, see Supplementary Fig. S6 for full-length blots. Gel images were processed and analyzed for quantification (Fiji, NIH). (**B**) Western blot showing increased phosphorylation of FLT3 among the c-KIT⁺ *MLL-AF9 knock in* cells in BM, after 4-weeks of HFD. After immunoprecipitation of pan tyrosine phosphorylated proteins with an anti-phosphotyrosine antibody (IP: pan Y), FLT3 was analyzed by a western blot (WB: FLT3). Increased phosphorylation of FLT3 following HFD was confirmed by immunoprecipitation with an anti-FLT3 antibody (IP: FLT3) followed by a western blot to detect pan phosphorylation of FLT3 (WB: pan Y). Data show mean ± SD; n = 3 mice per diet group; ***$P < 0.001$, two-tailed unpaired Student's t-test. Grouping of blots cropped from different gels, see Supplementary Fig. S7 for full-length blots. Gel images were processed and analyzed for quantification (Fiji, NIH). (**C**) Flow cytometry on *MLL-AF9 knock in* BM cells showing median fluorescence intensity (MFI) for expression of FLT3 on Lin⁻ SCA1⁺ c-KIT⁺ (LSK) or Lin⁻ c-KIT⁺ (LK) cells. Data were analyzed using FlowJo software (version 10, TreeStar Inc, https://www.flowjo.com). RTqPCR, performed on cell-sorted cells, showing no modulation in transcription of the *Flt3* gene among LSK or LK cells, following a HFD. Data show mean ± SD; n = 4 mice per diet group; *P* value measured by two-tailed unpaired Student's t-test; ns, non-significant. (**D**) Immunostaining showing that HFD induces cluster formation of lipid rafts (red), in which FLT3 (green) is typically condensed. Data on the left panel show examples of representative immunostaining for c-KIT⁺ cells from CD and HFD-fed mice. Microscopy is performed on c-KIT⁺ cells isolated by magnetic beads. Fluorescence was observed by microscopy (Axio Imager 2, Zeiss) and the images were processed (Fiji, NIH). White scale bar represents 5 µm. Arrows indicate FLT3/lipid raft clusters. Statistic is shown on the right panel. ***$P < 0.001$, two-tailed unpaired Student's t-test.

STAT3 ($P < 0.001$). However, we observed no disturbance in AKT phosphorylation, and no phosphorylation was detected for STAT5 (Fig. 3A).

The FMS-like tyrosine kinase-3 (FLT3) activates the JAK-STAT pathway, which leads to increased proliferation/survival of human AML cells[18–22]. Moreover, MLL-AF9 was found to cooperate with activated FLT3 signaling to accelerate AML development in various mouse models[23–25]. Following a pull-down of all tyrosine-phosphorylated proteins, FLT3 was found to be highly phosphorylated ($P < 0.001$) in c-KIT⁺ cells isolated from HFD-fed mice, 4 weeks after the regimen. This increased phosphorylation of FLT3 was furthermore confirmed by immunoprecipitation with an anti-FLT3 antibody followed by western blotting to detect pan phosphorylation of FLT3 ($P < 0.001$) (Fig. 3B).

In conclusion, HFD activated the FLT3 receptor, which enhanced the downstream JAK3/STAT3 signaling among c-KIT⁺ cells.

HFD increases clusterization of the FLT3 receptor within lipid rafts. As measured by flow cytometry, enhanced phosphorylation of FLT3 was not due to an increased expression of the FLT3 protein on the cell surface of the primitive HSPC from HFD-fed mice. In addition, we found no changes in *Flt3* gene transcription

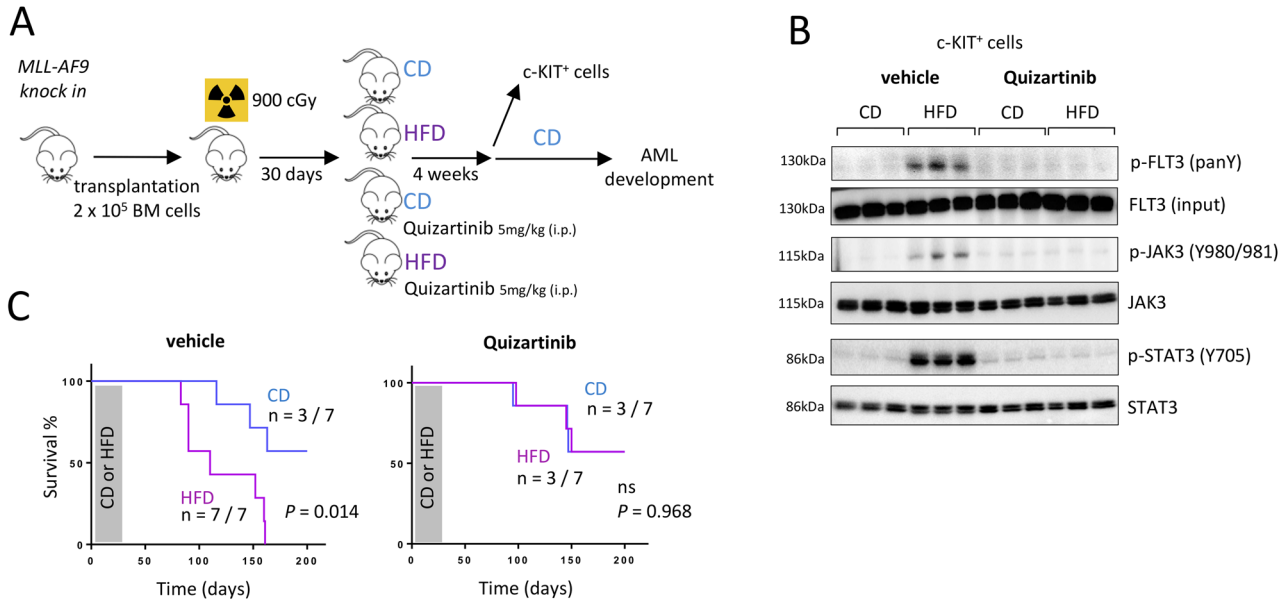

Figure 4. Quizartinib blocks the HFD-accelerated development of AML. (**A**) Experimental workflow describing the procedure. Image performed with the GIMP software (version 2.10.18, GIMP, https://www.gimp.org/news/2020/02/24/gimp-2-10-18-released/). (**B**) Western blot showing that a treatment with Quizartinib antagonizes the increased phosphorylation of FLT3 (pan tyrosine; panY) as well as JAK3 (Y980/981) and STAT3 (Y705) observed among the c-KIT⁺ *MLL-AF9 knock in* BM cells after 4-weeks of HFD. Data show mean ± SD; n = 3 mice per diet group. Grouping of blots cropped from different gels, see Supplementary Fig. S8 for full-length blots. Gel images were processed and analyzed for quantification (Fiji, NIH). (**C**) Survival curves showing that mice fed a HFD and treated with Quizartinib survived longer than CD-fed mice, n = 7 mice per group; *P* value measured by Mantel–Haenszel test.

in these cells (Fig. 3C). As platforms for membrane trafficking and signal transduction, lipid rafts are master regulators of cytokine function, and they regulate several important key receptors involved in hematopoiesis[10,26–31]. Lipid rafts are cholesterol-enriched patches located in the plasma membrane, and the dynamic protein assembly in these lipid rafts can be modified by a disturbance in the lipid composition of cells[10,28]. Through immunostaining, we discovered that, after only 4 weeks of a HFD regimen, the localization of FLT3 within lipid rafts changed on the cell surface of c-KIT⁺ cells isolated from BM. We detected that the number of cells showing FLT3/lipid rafts clusters increased markedly ($P < 0.001$) among the population of c-KIT⁺ cells from HFD-fed mice (Fig. 3D). After the mice had consumed the HFD for 4 weeks, increased phosphorylation of FLT3 was detected in primitive HSPC, but few weeks after the diet had stopped, while the HFD was withdrawn and mice were fed a CD until they developed AML, this phosphorylation was no longer observed in post-HFD AML cells (Supplementary Fig. S4A–B). Indeed, immunostaining did not show colocalization of FLT3 among lipid rafts, and no clusters were visualized on post-HFD AML cells (Supplementary Fig. S4C).

We can therefore conclude that a HFD regimen can transiently modify clusterization of the FLT3 receptor within lipid rafts on c-KIT⁺ cells.

Inhibition of the FLT3 phosphorylation blocks the HFD-enhanced development of AML. To confirm involvement of FLT3/JAK3/STAT3 signaling in the accelerated transformation of *MLL-AF9 knock-in* cells, we injected Quizartinib, a potent inhibitor of FLT3 tyrosine phosphorylation[32–34], showing efficiency between 1 to 10 mg/kg in vivo[34]. An intraperitoneal injection of 5 mg/kg was administered twice per week throughout the 4 weeks of the HFD (Fig. 4A). Four weeks after the beginning of the HFD, primitive HSPC expressed high phosphorylation/activation of the FLT3 receptor and showed active JAK/STAT signaling pathway. At the same time, c-KIT⁺ cells from mice that consumed the HFD and were treated with Quizartinib showed no activation of this pathway (Fig. 4B). When we examined mice for AML occurrence, HFD-fed mice developed AML much more quickly than did mice from the control group ($P = 0.014$). Meanwhile, HFD-fed mice treated with Quizartinib did not show an increased risk of developing AML ($P = 0.968$, Fig. 4C).

We can thus assume that the accelerated development of AML observed after consumption of a HFD is due to the increased activation of the FLT3 pathway.

Discussion

Several mouse models have been described in the literature to study the role of HFD and leukemias and all studies show that HFD accelerates progression and aggressiveness of the diseases. Following a HFD feeding on a mouse xenograft model to study acute leukemia, alteration of the metabolism has been shown in the tumor microenvironment, which has significantly impacted the leukemia development[35]. Growth of xenotransplanted myeloid leukemia cell lines was also influenced by HFD in vivo[36]. Using two murine models, HFD-induced obesity has

been shown to accelerate acute lymphoblastic leukemia progression[37]. The AML burden was also found much higher in HFD-induced obese mice, and the mechanism which link obesity with aggressive AML was due to an enhanced aberrant DNA methylation in AML cells[7,8]. All these findings, on several murine models used to study leukemias described more the role that obesity has on the tumor progression. In our study, we described for the first time, on a well-known murine model to study AML, that a HFD can accelerate the initiation of the *MLL-AF9*-driven AML disease. Furthermore, accelerated progression of AML has been described related to further mechanism, such as epigenetic which activate cell cycling[7,8], changes in the metabolism of leukemic cells[36,37], or in the microenvironment where cancer cells are expanding[35]. In our study, we described another mechanism, by which a HFD can modify the FLT3 signaling pathway known to accelerate the AML development in *MLL-AF9 knock-in* mouse model[24]. This mechanism involves that the HFD increases the clusterization of the FLT3 receptor within lipid rafts on the cell surface of primitive HSPC, which activates the phosphorylation of the FLT3 receptor, and can in turn triggers the stimulation of the downstream JAK3/STAT3 pathway in these cells.

FLT3 can be constitutively activated in human AML samples following either DNA duplication or mutations in the *FLT3* gene, which produce altered signaling[38–41]. Activated signaling in these models were consecutive to genetic modifications, such as expression of internal tandem duplications (ITD) or mutations in *FLT3*. On DNA extracted from post-HFD AML samples (n = 8 samples analyzed), we found no mutation or ITD in exons 14–15 of the *Flt3* gene encoding the juxtamembrane domain, and no mutation were found in exon 20 encoding the tyrosine kinase domain (TKD) in the *Flt3* gene (Supplementary Fig. S5). Therefore, the increased phosphorylation/activation of the FLT3 receptors and activated JAK/STAT signaling observed among primitive HSPC c-KIT$^+$ cells isolated from mice after 4 weeks of HFD cannot be due to a genetic alteration of the murine *Flt3* gene.

While STAT3 and STAT5 proteins were both oncogenic downstream mediators of the JAK/STAT pathway[42], STAT5 was actually shown as the target of constitutively active FLT3-ITD mutants, but not of the ligand-stimulated FLT3 wild-type receptor[43,44]. In our study, while no genetic alteration was detected in the *Flt3* gene, HFD overactivated the wild-type FLT3 receptor, and therefore only phosphorylation of STAT3 was found activated and no phosphorylation was detected for STAT5.

In animal model to study FLT3-ITD, Quizartinib has been described as a potent and selective inhibitor of FLT3 to treat mice bearing human AML xenografts[34], in which there is a constitutive phosphorylation of FLT3 due to the ITD. In our study focusing on leukemia initiation, we have transiently fed mice a HFD, therefore active phosphorylation of the wild-type FLT3 receptor was observed following only the 4 weeks of HFD. This result highlights that HFD, even during a short period, can turn on the phosphorylation of FLT3, which increases the initiation of *MLL-AF9*-induced AML. While after this regimen mice were fed a CD until they developed AML, there was no active phosphorylation of FLT3 observed in post-HFD AML samples (Supplementary Fig. S4B). Indeed, it does not seem judicious to us to treat post-HFD AML mice with Quizartinib, due to the absence of overactivation of FLT3 in post-HFD AML, this treatment should not be able to reduce the leukemia progression.

Conclusion

In summary, we show for the first time that a HFD can overactivate the FLT3 receptor on primitive hematopoietic cells, as well as the downstream JAK/STAT signaling known to accelerate transformation of *MLL-AF9 knock-in* BM cells. Treatment of mice with Quizartinib inhibited FLT3 phosphorylation on primitive hematopoietic cells, which resulted in the blockade of the JAK3 and STAT3 phosphorylation that typically occurred during the HFD regimen, and finally antagonized the initiation of AML and its accelerated development. We can therefore conclude that a HFD regimen enforced the expression of FLT3 signaling and enhanced the oncogenic transformation of *MLL-AF9 knock-in* BM cells. Using a mouse model of AML, we revealed that consuming a HFD, even in the short term, accelerates the risk of leukemia development.

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Author contributions

F.H. performed experiments, analyzed data, discussed the project and helped in writing the manuscript. R.M. and J.S. performed the western blot experiments. P.C. helped for performing the CGH Array experiments and discussed the project. L.D. discussed the project. R.Q. conceived the project, performed experiments, analyzed data, discussed the project and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Title: Study of the effect of *HSP90* inhibitors in the treatment of T-ALL and B-ALL, by suppressing the *LCK* and *LYN* signaling pathways

Key words: *Acute T-cell and B-cell Lymphoblastic Leukemias, HSP90 Inhibitors, NVP-BEP800, LCK signaling pathway, LYN signaling pathway.*

Abstract: My PhD work relates to T-cell and B-cell acute lymphoblastic leukemia. This project allowed us to identify a novel therapeutic strategy to treat ALL, by using an HSP90 inhibitor, that suppresses *LCK* and *LYN* pathways in T-ALL and B-ALL. Due to the crucial role of both *HSP90* and *SRC* kinases in tumor development, several molecules that inhibit *SRC* or *HSP90* have been developed in order to block cancer cell proliferation.

Our work showed that *HSP90* is overexpressed in ALL cells compared to resting B and T cells. Moreover, we showed that *HSP90* overexpression is accompanied by an overexpression of *LYN* kinase in B-ALL and *LCK* kinase in T-ALL. we found that HSP90 is an important regulator of *SRC* kinases(*LCK* and *LYN*), which are involved in the intracellular signaling pathways required for the growth and proliferation of T-ALL, B-ALL and other types of leukemic cells. The inhibition of *HSP90* by NVP-BEP800 induces dissociation of the aberrant *HSP90-LYN* complex in B-ALL cells and disrupts the entire BCR signaling pathway. *LYN* and *NFκB* lose phosphorylation and become inactive, and the latter leave the nucleus, which leads to inhibition of the survival, growth and maintenance of B-ALL cells. We also showed that treating T-ALL cells ,with NVP-BEP800, leads to the *calcium / NFAT* pathway inactivation, and *LCK* dephosphorylation, whereas *NFAT1* becomes phosphorylated (inactive) and leaves the nucleus leading to inhibition of survival, growth and maintenance of T-ALL cells. These results were published in Blood Cancer Journal in March 2021.

Titre : Étude de l'effet des inhibiteurs de *HSP90* dans le traitement des leucémies lymphoïdes aiguës T et B, en inhibant les voies signalétiques *LCK* et *LYN*

Mots clés : *Leucémies Aiguës Lymphoblastiques T et B, Inhibiteurs d'HSP90, NVP-BEP800, Voie signalétique LCK, Voie signalétique LYN.*

Résumé : Mon travail de thèse porte sur les leucémies aiguës lymphoblastiques T et B. Ce projet nous a permis d'identifier une nouvelle stratégie thérapeutique pour traiter différentes types de LAL, en utilisant un inhibiteur *HSP90*, qui inhibe les voies signalétiques *LCK* et *LYN* importantes dans les LAL-T et LAL-B. En raison du rôle critique des kinases *HSP90* et *Src* dans la progression tumorale, plusieurs molécules inhibitrices de *SRC* ou *HSP90* ont été développées afin de bloquer la prolifération des cellules cancéreuses.

Nos travaux ont montré que *HSP90* est surexprimé dans les cellules LALs par rapport aux cellules B et T contrôles. De plus, nous avons montré que la surexpression de HSP90 s'accompagne d'une surexpression de la *LYN* kinase dans la LAL-B et de la *LCK* kinase dans la LAL-T. nous avons constaté que *HSP90* est un régulateur important des kinases *SRC (LCK* et *LYN*), qui sont impliquées dans les voies de signalisation intracellulaires nécessaires à la croissance et à la prolifération des cellules LAL-T, LAL-B et d'autres types de cellules leucémiques. L'inhibition de *HSP90* par NVP-BEP800 induit la dissociation du complexe aberrant *HSP90-LYN* dans les cellules LAL-B et perturbe l'ensemble de la voie de signalisation "B-cell receptor (BCR)". *LYN* et *NFκB* perdent leur phosphorylation et deviennent inactifs, et le dernier quitte le noyau, ce qui conduit à une inhibition de la survie, de la croissance et du maintien des cellules LAL-B. Nous avons également montré que le traitement des cellules LAL-T, avec NVP-BEP800, conduit à l'inactivation de la voie *calcium / NFAT* et à la déphosphorylation de *LCK*, alors que *NFAT1* devient phosphorylé (inactif) et laisse le noyau entraînant une inhibition de la survie, de la croissance et du maintien des cellules LAL-T. Ces résultats ont été publiés dans Blood Cancer Journal en Mars 2021.