B-cell receptor signalling and acquired resistance to chronic Src-family kinase inhibition in diffuse large B-cell lymphoma

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DEDICATION

To my mother and grandmother, two pillars of my life.
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LIST OF ABBREVIATIONS

CARD11: Caspase recruitment domain-containing protein 11

BCL-10: B-cell lymphoma/leukemia 10

MALT1: mucosa-associated lymphoid tissue lymphoma translocation protein 1

LMO2: LIM domain only 2

LRMP: lymphoid-restricted membrane protein.

DLBCL: Diffuse large B-cell lymphoma

NHL: non-Hodgkin lymphoma

GCB: germinal center B-cell

ABC: activated B-cell

BCR: B-cell receptor

GELA: Groupe d'Etude des Lymphomes de l'Adulte

R-CHOP: combination therapy including rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone

PFS: progression-free survival

OS: overall survival

EFS: event-free survival

ASCT: autologous stem cell transplantation

DNA: Deoxyribonucleic acid

BL: Burkitt lymphoma
AID0: activation-induced cytidine deaminase

PMBCL: primary mediastinal B-cell lymphoma

LLMPP: Lymphoma/Leukemia Molecular profiling project

EZH2: Enhancer of zeste homolog 2

PTEN: Phosphatase and tensin homolog

BCL6: B-cell lymphoma 6 protein

CDKN1A: cyclin-dependent kinase inhibitor 1A

PI3K: Phosphatidylinositol-4, 5-bisphosphate 3-kinase

AKT: Protein kinase B

mTOR: mammalian target of rapamycin

4E-BP1: Eukaryotic translation initiation factor 4E-binding protein 1

XBP1: X-box binding protein 1

TLR: Toll-like receptors

NF-kB: nuclear factor-κB

MYD88: Myeloid differentiation primary response gene 88

TNFAIP3: Tumor necrosis factor α-induced protein 3

SRC: Proto-oncogene tyrosine-protein kinase Src

BTK: Bruton's tyrosine kinase

SYK: spleen tyrosine kinase

ITAM: immunoreceptor tyrosine-based activation motif
LYN: Tyrosine-protein kinase Lyn
FYN: Proto-oncogene tyrosine-protein kinase Fyn
HCK: Tyrosine-protein kinase HCK
BLK: B lymphocyte kinase
BLNK: B-cell linker protein
CIN85: Cbl-interacting protein of 85kDa
PLCγ2: phospholipase Cγ2
DAG: diacylglycerol
IP₃: inositol trisphosphate
PIP₃: phosphatidylinositol-3, 4, 5-triphosphate
MAPK: mitogen-activated protein kinase
NFAT: nuclear factor of activated T cells
BCAP: B-cell adaptor for phosphoinositide-3-kinase
GAB 1/2: GRB2-associated-binding protein 1 and 2
SH2: Src Homology 2 domain
SH3: Src Homology 3 domain
Cbp/PAG1: Phosphoprotein associated with glycosphingolipid-enriched microdomains 1
SHP-1: Tyrosine-protein phosphatase non-receptor type 6
SHP-2: Tyrosine-protein phosphatase non-receptor type 11
FcγRIIB9: Fc fragment of IgG low affinity IIb receptor

ZAP70: related protein ζ-chain-associated protein kinase of 70 kDa

PTK: Protein tyrosine kinases

TKI: tyrosine kinase inhibitors

CML: chronic myeloid leukemia

FDA: Food and Drug Administration

CLL: chronic lymphocytic leukemia

MCL: mantle cell lymphoma

CRISPR: clustered regularly interspaced short palindromic repeats
ABSTRACT

In many lymphoid malignancies acknowledgment of the oncogenic role of B cell receptor (BCR) signaling led to the preclinical and clinical testing of tyrosine kinase inhibitors targeting this pathway. Although seminal studies suggested dependency on chronic BCR signaling in the ABC-DLBCL subtype, recent reports suggest the involvement of tonic BCR signaling in the GCB DLBCL subtype. For my M.Sc. project, I used a GCB DLBCL cell line (OCI-LY1), inhibitors of BTK, SFKs and PI3K signaling to evaluate the role of BCR signaling in proliferation and survival. I assessed for targetable changes in oncogenic addiction and BCR signaling in OCI-LY1 cells chronically exposed to dasatinib. The role of LYN in these processes was measured by knocking out the gene using CRISPR-Cas9 technology. Additionally, we generated dasatinib resistant cells that were 30 fold more resistant to dasatinib and four fold more resistant to ibrutinib. Our results suggest that tonic BCR signaling contributes to proliferation and survival in parental OCI-Ly1 cells. Our results suggest as well that LYN in addition to being a major target of dasatinib in these cells, it also contributes to BTK activation since lyn KO-OCI-Ly1 were more sensitive to both, dasatinib and ibrutinib. Altogether, our results support the role of LYN in the survival of germinal center B-cell like DLBCL cells and they contribute in the understanding of this important modulator of BCR signaling.
ABRÉGÉ
Dans un grand nombre de tumeurs lymphoïdes malignes, les avances oncogéniques impliquant la voie de signalisation BCR ont conduit à des essais précliniques et puis-clinique utilisant des inhibiteurs de tyrosine kinases ciblant cette voie. Bien que les études préliminaires aient suggéré la dépendance de la voie de signalisation chronique de BCR dans le sous-type LDGCB ABC, des articles récents suggèrent l'implication de signalisation tonique de BCR dans le sous-type LDGCB GCB. Dans le cadre de recherche pour ma maîtrise j'ai utilisé une lignée cellulaire LDGCB GCB (OCI-LY1), mais aussi des inhibiteurs de BTK, SFKs et de la voie de signalisation de PI3K pour évaluer le rôle de la signalisation de BCR dans la prolifération et la survie de ces cellules. De plus, j'ai ciblé les modifications impliqué dans la dépendance et la signalisation oncogénique de BCR dans les cellules OCI-LY1 exposés au long courir au dasatinib. La contribution de LYN à ces processus a été évaluée en éteignant le gène grâce à la technologie CRISPR-Cas9. En outre, nous avons généré des cellules résistantes au dasatinib stables qui étaient 30 fois plus résistantes au dasatinib et quatre fois plus résistantes à l'ibrutinib. De plus, nos résultats suggèrent que LYN en plus d'être une cible majeure de dasatinib dans ces cellules, contribue également à l'activation de BTK puisque les cellules Lyn KO-OCI-Ly1 cellules étaient plus sensibles à la fois au dasatinib et l'ibrutinib. Au total, nos résultats confirment le rôle de LYN dans la survie des cellules LDGCB GCB cellules et
contribuent à améliorer la compréhension de ce modulateur majeur de la signalisation de la voie BCR.
1 Introduction.

1.1 DLBCL

Lymphoma is a disorder of lymphocytes that arises in secondary lymphoid organs or extranodal tissues[1]. Diffuse large B cell lymphoma (DLBCL) is the most common malignant subtype of non-Hodgkin lymphoma (NHL), accounting for around 40% of all newly diagnosed lymphomas [2, 3]. It is considered an aggressive lymphoma, characterized by rapid growth and limited survival in the absence of treatment. Although around 50% of cases can be cured, up to one-third of patients eventually relapse or fail to achieve remission [4, 5].

1.2 DLBCL treatment

DLBCL was initially treated with combination chemotherapy in the 1970s through the use of anthracycline–based regimens [6] followed by the use of the combination of cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) in the 1990s [5, 7]. A decade later a big step forward in the treatment of DLBCL was achieved by the incorporation of rituximab, a chimeric monoclonal antibody that targets the CD20 receptor present in B cells. Results from several clinical trials performed in the early 2000s established R-CHOP as the de facto front-line treatment for patients of all ages with DLBCL [5, 8-11].

In the past decade, a better understanding of the pathobiology of DLBCL together with technological advances has led to the development of novel agents with greater
specificity and lower generalized toxicity than first-line treatments. The clinical efficacy of several tyrosine kinase inhibitors in DLBCL are being tested in ongoing clinical trials. Promising preliminary results indicate that DLBCL therapy is undergoing a paradigm shift away from conventional therapy towards personalized medicine [12-25]. In the following sections, the characterization of DLBCL at the molecular level together with recent advances in the development of targeted small molecule inhibitors are reviewed.

1.3 Germinal-Center and DLBCL Sub-types

The pathogenesis of B-cell lymphoma is directly connected with the differentiation stage of the B-cell clone that gives rise to the malignancy. The proliferation and differentiation of B cells is strictly linked with the function and maturation of the B-cell receptor (BCR) [26-28]. The variability of the immunoglobulin component of the BCR and the ability of a selected B cell to clonally proliferate in response to infection are the pillars of adaptive immunity. This process involves DNA strand breaks, DNA mutations, loss of genetic material and clonal cell proliferation.

Although this defense mechanism provides essential protection for the organism, deregulation of this unique molecular pathway might results in the development of lymphoid malignancies [27]. Because these processes are triggered by the activation of mature naïve B cell at the germinal centers (GCs), these sites have been proposed to be the source of many lymphomas types, particularly follicular lymphoma, Burkitt lymphoma
(BL) and diffuse large B-cell lymphoma (DLBCL) [2, 27, 29]. During the germinal-center reaction, the B-cell receptor is altered by two distinct modifications of the B-cell DNA: class-switch recombination and somatic hypermutation, both of which need activation-induced cytidine deaminase (AID) activity. Class-switch recombination allows for a change in the immunoglobulin heavy-chain class from IgM to IgG, IgE or IgA, while somatic hypermutation involves immunoglobulin-variable-region mutations, which generates a population of B cells with increased (or reduced) affinity for a particular antigen [2, 30].

Although DLBCL cells carry the differentiation program of normal B cells from which they arise, oncogenic abnormalities in their genome disrupt the normal program. As a result, malignant B cells manage to avoid apoptosis, block plasma differentiation and display deregulated pathways of survival and cell growth [2-5, 31]. Besides the fact that DLBCL has a significant molecular heterogeneity and is clinically diverse, until recently it was not possible to subdivide it into distinct disease entities due to overlapping morphology and pathologic features.

Based on the putative cell of origin and through large-scale gene-expression profiling (GEP) morphological indistinguishable DLBCL tumors now can be subdivided into three distinct cell-of-origin subtypes: germinal center B-cell (GCB), activated B-cell (ABC) and primary mediastinal B-cell lymphoma (PMBCL), [Figure 1][5, 31-37]. These
subtypes arise from germinal center B cells that are at distinct stages of differentiation and therefore they differ in the expression of thousands of genes. Additionally, they diverge in clinical presentation, response to targeted regimens and overall survival rate after chemotherapy[2]. Several clinical studies demonstrated that patients with GCB DLBCL have a better prognoses than those with ABC DLBCL [5, 38, 39].

Figure 1. Stratification of DLBCL into subtypes through gene expression profiling. Adapted and reprinted from Weinberg, 2014 [37]. Copyright permission granted from Garland Science.
GCB DLBCL are thought to derive from lymphoid cells located in the germinal center and therefore they express germinal center signature genes such as CD10, LMO2, LRMP and the transcription repressor BCL6 [3, 5, 32]. Around 30-40% of GCB DLBCL cases have a t(14-18) translocation, 30% present a c-rel amplification, 20% harbor mutation of the histone methyltransferase EZH2, 10% have a deletion of the tumor suppressor PTEN and between 6-12% have an amplification of the microRNA cluster miR-17-92 (which targets PTEN); all these genetic abnormalities are almost never observed in the ABC subtype. EZH2 is a master regulator of the GCB subtype and cooperates with BCL6 to mediate lymphomagenesis in GCB DLBCL. A recurring somatic point mutation within exon 5 leads to gain of function and the increased methylation of histone 3, which might induce lymphomagenesis through transcriptional silencing of key antiproliferative and tumor suppressor genes involved in cell cycle regulation, such as CDKN1A [3, 5, 40-44]. Selective EZH2 inhibitors such as GSK126 and E11 showed a decrease in proliferation and induction of cell cycle arrest and apoptosis in DLBCL cell lines and xenograft mouse models [5, 45, 46]. The activation of the phosphatidylinositol 3 kinase (PI3K)/AKT/mTOR pathway is important for cellular growth, proliferation and metabolism, and has been recently associated with several B-cell lymphomas, including GCB DLBCL [3, 47-50]. Although deletion of PTEN, the tumor suppressor gene involved in the negative regulation of this signaling, has been detected in just 10% of GCB DLBCL, the loss of PTEN expression by immunohistochemistry (IHC) was observed in around 55% of GCB DLBCL
PTEN status was inversely correlated PI3K/Akt pathway activation in GCB DLBCL cell lines, suggesting a constitutive activation of the pathway and an oncogenic addiction specific for the GCB subtype [5, 48, 49]. Inhibitors of the PI3K/Akt/mTOR axis are under development and some are undergoing early phase clinical testing in all subtypes of relapsed DLBCL [5, 12-17]. Another amenable therapeutic target in GCB DLBCL is the apoptotic regulator B-cell lymphoma 2 (BCL-2). The BCL-2 gene is deregulated in DLBCL through several mechanisms that diverge among the molecular subtypes. The BCL-2 translocation t (14; 18) is found in between 34-45% of GCB DLBCL cases and next-generation sequencing data has shown that BCL-2 is the most mutated gene in GCB DLBCL [5, 51]. ABT-199, a second generation inhibitor of BCL-2 has demonstrated promising activity in DLBCL patients and is currently being tested in a phase II study in patients with relapsed DLBCL of all subtypes [5, 18].

**ABC DLBCL** are believed to derive from B-cells at a plasmablastic stage, just before exiting the germinal center, as a consequence ABC lymphomas have the plasma-cell gene expression program, including the master regulator of immunoglobulin secretion: transcription factor XBP1 [2, 52, 53]. Numerous genetic abnormalities in the ABC subtype are rare in the other subtypes, most of ABC lymphomas overexpress BCL-2 and several amplify the BCL-2 locus [2, 54]. A unique characteristic of B cells is the expression of both an antigen-specific B-cell receptor (BCR) and one or more receptors of the innate
immune system, known as Toll-like receptors (TLR). This dual receptor pattern allows B cells to display an integrated response to different stimuli and promote the activation of downstream transcription factors, such as the NF-kB signaling[5]. The pathogenic hallmark of ABC DLBC is the constitutive activation of the NF-kB pathway, which promotes: proliferation, cell survival, cytokine secretion, angiogenesis, invasion, inhibition of apoptosis and metastasis. This aberrant activation is mainly due to constitutive activation of the CBM signaling complex (composed of CARD11, BCL-10 and MALT1) [2, 3, 5].

Several genetic abnormalities can activate the CBM complex; around 10 % of ABC cases harbor activating mutations of CARD11 and mutant CARD11 isoforms form protein aggregates in the cytoplasm where NF-kB constitutive activation occurs [2]. Many ABC DLBCL cases have wild-type CARD11, nevertheless they still rely on CARD11 for NF-kB activation. In these cases NF-kB triggering is mediated by chronic activation of the B-cell receptor signaling and downstream tyrosine kinase activity, which then leads to an engagement of the CBM pathway. Around 20% of ABC cases harbor mutations in the BCR signaling sub-units CD79a and CD79b, the mutant CD79 proteins increase B-cell receptor expression and reduce activation of LYN, a negative regulator of BCR signaling [2, 55]. Recurrent oncogenic mutations in MYD88, which encodes an adaptor protein involved in Toll-like receptor signaling (TLR), have been reported in almost 30% of ABC DLBCL cases. MYD88 mutations activate a complex of IL-1-associated kinases: IRAK-1
and IRAK-4, which then allows the engagement of NF-kB and MAP kinase signaling [5, 56]. Tumor necrosis factor α-induced protein 3 (TNFAIP3), also known as A20, is a negative regulator of NF-kB signaling. In around 30% of ABC DLBCL cases, biallelic inactivation of TNFAIP3 due to mutations can coexist with mutation in CD79a/CD79b and MYD88, these observations suggest that inactivation of TNFAIP3 can enhance both the BCR and TLR signaling pathways [5, 57-60]. The most common genetic alterations reported in the GCB and ABC subtypes is summarized in figure 2.

Figure 2. Genetic alterations found in GCB and ABC DLBCL subtypes. This figure is adapted and reprinted from an article published by Pasqualucci and Dalla-Favera, 2014 [51]. Copyright permission granted from Elsevier.
1.4 BCR signalling

B-cell receptor (BCR) signaling has emerged as an important oncogenic pathway which promotes growth and survival in several lymphoma subtypes [1]. The ABC subtype is dependent for survival on NF-κB activation attributed to chronic BCR and TLR signalling [1, 16, and 57]. In contrast, recent reports suggest involvement of the PI3K pathway triggered by tonic BCR activity in GCB DLBCL [1, 5, and 16, 36-38, 57-59]. The initiation and amplification of BCR signaling is determined by the efficiency of the recruitment and activation of the Src, BTK and SYK family kinases to the BCR complex [16].

Normal B cells and every B-cell lymphoma cell has a pair of heavy- and light-chain immunoglobulins that are the result of rearrangement of immunoglobulin subgenes. Consequently each BCR has a distinctive antigen-binding site. The surface immunoglobulins of the BCR are non-covalent coupled with the heterodimer of CD79A (Igα) and CD79B (Igβ) proteins. Both CD79A and CD79B subunits contain a single immunoreceptor tyrosine-based activation motif (ITAM) which is composed of two tyrosine residues [27]. Antigen-induced aggregation of the BCR lead to phosphorylation of the ITAMs by the Src-family kinases, including LYN, FYN,HCK and BLK [1]. The dual phosphorylation of the ITAMS creates docking sites that allows the recruitment of the tyrosine kinase SYK through its tandem Src homology 2 (SH2) domains, resulting in SYK phosphorylation and activation [1, 27]. The Src-family kinases and SYK trigger a signalosome composed of multiple kinases and adaptor proteins. SYK recruits a complex
of B-cell linker protein (BLNK) and Cbl-interacting protein of 85kDa (CIN85). This complex in turn coordinates the phosphorylation and subsequent activation of Bruton tyrosine kinase (BTK) and phospholipase Cγ2 (PLCγ2) [1, 61]. PLCγ2 then catalyzes the hydrolysis of phosphatidylinositol-4, 5-bisphosphate (PI (4, 5) P2) into diacylglycerol (DAG) and inositol trisphosphate (IP3), causing mobilization of calcium ions [1, 26]. The combination of increased intracellular calcium and DAG activates protein kinase Cβ (PKCβ), which in turns mediates the activation of the nuclear factor-κB (NF-κB) pathway [1, 62]. An important adaptor involved in the BCR signalosome is the co-receptor protein CD19. The phosphorylation of CD19 by the Src-family kinase LYN leads to the recruitment of phosphoinositide 3-kinase (PI3K) to the BCR. PI3K then phosphorylates PI (4, 5) P2 to generate phosphatidylinositol-3, 4, 5-triphosphate (PIP3), which in turns allows the recruitment of BTK and AKT to the cell membrane. The final result of proximal BCR signaling is the initiation of NF-κB, PI3K/mTOR, mitogen-activated protein kinase (MAPK), nuclear factor of activated T cells (NFAT) and RAS pathways, which promotes proliferation, survival, metabolic changes and migration of normal and malignant B cells (Fig.3) [1, 26, 27, 63].
Figure 3. B-cell receptor signaling and downstream pathways involved in survival and proliferation. This figure is adapted and reprinted from an article published by Young, 2013 [1]. Copyright permission granted from Nature Publishing Group.

1.5 Tonic BCR and PI3K/mTOR signaling in GCB DLBCL

In contrast to antigen-dependent BCR signaling that initiates the germinal centre response, mature B cells utilize the BCR in a distinct fashion known as tonic BCR signaling, a process that is required for B-cell survival and is believed to be ligand-independent [1, 26, 27]. Evidence for this comes from experiments with mature mouse B cells in which CD79a and IgM were inactivated, resulting in a severe reduction of peripheral B cells. Provision of a construct with CD79a with a truncated ITAM was unable to rescue the CD79a-deficient B cells, indicating the essential role of BCR signaling in B
cell survival [1, 64, 65]. In follow up studies constitutively active PI3K/AKT signaling, but not NF-κB or MAPK signaling, rescued the survival of BCR-deficient B cells, these results demonstrated the importance of PI3K/Akt pathway in tonic BCR signaling (Fig.4)[1, 66].

Figure 4. Tonic BCR signaling engaging PI3K/mTOR axis. This figure is adapted and reprinted from an article published by Young, 2013 [1]. Copyright permission granted from Nature Publishing Group.
PI3K binding in the plasma membrane is mediated by its association with docking and adaptor proteins including: co-receptor CD19, B-cell adaptor for phosphoinositide-3-kinase (BCAP), GRB2-associated-binding protein 1 and 2 (GAB1/2) [27, 67, 68]. These adaptor molecules serve in the assembling of the BCR signalosome and its signal amplification, however their functional meaning in the apparent redundancy in PI3K recruitment is unknown [27]. CD19 and BCAP, which are phosphorylated by the kinases LYN and SYK, co-aggregate with the BCR complex and directly bind the PI3K sub-unit p85, which in turns allows PI3K activation in the plasma membrane [68-73]. How PI3K signaling is initiated in the ligand-independent BCR signaling is still under debate. However, a model suggests the involvement of the GTPase TC21 (also known as R-RAS2), which promotes PI3K activity by presumably recruiting the catalytic sub-unit p110δ to the BCR complex resulting in tonic signaling [74].

1.6 LYN Kinase

Among the Src-family kinases involved in BCR signaling LYN is predominant and its importance relies on its dual role in the regulation of this signalosome. LYN is regulated by protein interactions through its SH2/SH3 domains as well as via its phosphorylation status [75]. At its carboxyl terminus LYN is phosphorylated by Csk, resulting in an inactive state of the kinase. In contrast, activation of LYN involves dephosphorylation of the C-terminal tyrosine (Y508) by phosphatases such as CD45 and SHP-2, which release the
inhibitory configuration of the kinase domain. LYN can then trans-autophosphorylate its activation tyrosine (Y396) to become a highly active enzyme (Fig.5) [76].

Figure 5. LYN structure and regulation of active and inactive kinase state. This figure is adapted and reprinted from an article published by Xu et al, 2005 [77]. Copyright permission granted from Elsevier Inc.

In the inactivation cycle LYN is further controlled by interactions with adapter and/or scaffold proteins that are spatially confined to specific cellular compartments, such as, Cbp/PAG1 which is localized in lipid rafts of the cell membrane [78-80]. Other negative regulators of LYN are the phosphatases SHP-1 and SHP-2, which are themselves attracted to receptors phosphorylated by LYN. These phosphatases are known to dephosphorylate the pY397 activation loop site, therefore they are critical players in the negative regulation of the kinase [81, 82].
LYN is important for positive regulation of BCR signaling through phosphorylation of the ITAMS in the BCR sub-units CD79a, CD79b and the co-receptor CD19, and this role apparently can be assumed by the other SFKs involved in the BCR signaling such as FYN, HCK and BLK. However, its unique role in the negative regulation of BCR signaling resides in its ability to phosphorylate immunoreceptor tyrosine-based inhibition motifs (ITIMs) in inhibitory cell surface receptors such as FcγRIIB9 (CD32b), PIR-B, CD5 and CD22 [77, 81, 83]. The phosphorylated ITIMs allowed the recruitment of inhibitory phosphatases such as SHP-1/2 and SHIP-1, which down modulate the signaling.

In addition, LYN is important for the recruitment of CSK into the lipids rafts of the plasma membrane through tyrosine phosphorylation of Cbp/PAG, resulting in down regulation of the BCR signaling as well. Generation of lyn-/- mice and the finding of their predisposition to autoimmune disease and B-cell hyper responsiveness revealed a negative regulatory role for LYN in BCR signaling [77]. Furthermore, experiments in vitro with lyn-/- primary B cells showed an increase in MAPK, enhanced calcium flux and hyper activation of AKT, altogether these experiments proved the critical role of LYN in the negative feedback control of BCR signaling [77, 79, 81, 83, 84].

Regarding a potential role of LYN in cancer, studies in knockout mice suggest that LYN can play an oncogenic role in neoplastic cells, moreover, mounting evidence strongly implicates a role for LYN in several types of leukemia and lymphoma [85-89]. In addition,
several studies showed a direct link between LYN activity and PI3K/Akt signaling mediated by the recruitment of the p85 subunit to phosphorylated tyrosine’s within docking and adapter proteins such as CD19, BCAP and Gab1 [1, 27, 68, 70-72, 84].

These observations highlight the importance of studying the role of LYN tyrosine kinase activity in the cancer biology of GCB DLBCL and the cross talk between BCR and PI3K/Akt signaling. Figure 6 illustrates a schematic representation of pathways and substrates modulated by LYN.

Figure 6. Positive and negative signaling modulated by LYN. This figure is adapted and reprinted from an article published by Xu et al, 2005 [77]. Copyright permission granted from Elsevier Inc.
1.7 SYK Kinase

Dual-phosphorylation of ITAMs within immunoreceptors such as the B-cell receptor (BCR), T-cell receptor and Fc receptor (FcRs) and interactions with their SH2 domains, leads to the recruitment and activation of spleen tyrosine kinase (SYK) or the related protein ζ-chain-associated protein kinase of 70 kDa (ZAP70), this kinase activation in the receptor complex triggers downstream signaling through phosphatidylinositol-3-kinase (PI3K), NF-κB, extracellular signal-related kinase (ERK)-mitogen-activated protein kinase (MAPK), and NFAT pathways. [90, 91].

SYK’s pivotal role in mediating inflammatory responses combined with its recently reported involvement in malignancy has made this kinase in an amenable target for the development of therapeutic agents [92]. A study of 69 DLBCL tumor samples from human patients reported that around 44 % had elevated levels of phosphorylated SYK, which was localized in proximity to BCR complexes in the plasma membrane [93]. SYK inhibitors such as R406 or PRT318 are either cytotoxic or cytostatic for most DLBCL cells which display a BCR in the membrane, moreover, SYK knockdown with siRNA arrested cell cycle progression in DLBCL cell lines [93, 94]. Altogether, mounting evidence indicates that SYK is an important mediator of chronic and tonic BCR signaling and its role is critical for survival and proliferation of malignant DLBCL cells [95].
1.8 Tyrosine kinase inhibitors

An increase in the understanding of the physiology of lymphoid malignancies has revealed new targets including components of the immune system and cell-signaling pathways, which opens an opportunity that can be exploit for therapy of these diseases. Protein tyrosine kinases (PTK) are ideal candidates in approaches targeting signal transduction and this is based on their roles in the signaling and the pathogenesis of the disease. In addition, the fact that PTK are enzymes makes them even more attractive as targets since they can be turned-off by drugs that block their catalytic sites [96]. There are over 100 PTK which play important roles in survival, proliferation, response to chemotherapy and migration of cancer cells [96]. Because of their role in regulating these functions in cancer cells the therapeutic efficacy of tyrosine kinase inhibitors (TKI)s has been studied extensively. The first TKI introduced in clinic was imatinib mesylate, which was used to treat the chronic phase of chronic myeloid leukemia (CML). The commercial version (Gleevec®, Novartis) was approved by the FDA in 2001 as first line therapy in CML. Evidence of resistance to imatinib in a subgroup of patients led to development of second generation TKIs such as dasatinib.

1.9 Dasatinib

Dasatinib (Sprycel®, Bristol Myers Squibb) is a potent multitarget kinase inhibitor of BCR-ABL and Src-family kinases, it can inhibit proliferation of wild-type and BCR-ABL mutant cell lines resistant to imatinib. Studies in vivo have shown that dasatinib is 325-
fold more potent than imatinib. In 2006, the FDA approved dasatinib for the treatment of chronic phase, accelerated phase or blastic phase CML, resistant or intolerant to imatinib and for Philadelphia chromosome-positive acute lymphoid leukemia (ALL) that is resistant to prior therapy [97-99].

Figure 7. Molecular structure of Dasatinib. This figure is adapted and reprinted from an article published by Weisberg et al, 2007 [100]. Copyright permission granted from Nature Publishing Group.

In 2008, a group at Cornell University reported that dasatinib blocked cell cycle progression at the G1-S phase transition in DLBCL cell lines and inhibited B-cell receptor signaling in primary lymphoma cells, suggesting a potential use of the compound in DLBCL therapy [101]. In 2010 our group showed that dasatinib decreased ligand dependent calcium mobilization through a decrease in BCR signaling in GCB-DLBCL cell
lines [102]. These two independents investigations showed the great potential in targeting BCR signaling in DLBCL through Src-family kinase inhibition.

### 1.10 Ibrutinib

Bruton’s tyrosine kinase (BTK) is an important component of BCR signaling and has been implicated in the development of multiple B cell malignancies including chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), follicular lymphoma and diffuse large B cell lymphoma (DLBCL) [103, 104]. Ibrutinib (Imbruvica TM) is a small molecule first-in-class developed by Pharmacyclics Inc. and Janssen Biotech Inc. that covalently binds to the cysteine-481 amino acid of BTK, rendering it inactive [105-107].

In November of 2013 Ibrutinib was granted an accelerated approval by the FDA for the treatment of relapsed mantle cell lymphoma and later in February of 2014 for refractory chronic lymphocytic leukemia (CLL) [106, 108]. In DLBCL, Ibrutinib showed selective toxicity in cell lines with chronic active BCR signaling [55] and preclinical studies with lymphoma canine models showed that Ibrutinib occupied BTK in blood and tumour tissues [106].

Published data [104, 106, 107, 109, 110] and conference presentations of ongoing clinical trials highlight the promising activity of Ibrutinib in DLBCL and other B cell malignancies. Further research is necessary for clarification of the role of BTK in tonic BCR signaling and its potential use in GCB DLBCL.
Figure 8. Molecular Structure of Ibrutinib and proposed binding model showing the covalently bound residue Cys-481. This figure is adapted and reprinted from an article published by Burger and Buggy, 2013. Copyright permission granted from Informa UK, Ltd.

1.11 PI3K/mTOR Inhibition

The signaling network comprised by phosphoinositide 3-kinase (PI3K), AKT and mammalian target of rapamycin (mTOR) regulates most hallmarks of cancer which include: survival, cell cycle, metabolism and genomic instability [111]. Studies in cancer genetics suggests that the PI3K signaling is the most frequent altered pathway in human tumours. Two players involved in the deregulation of this pathway are: 1) the PIK3CA gene that encodes the PI3K catalytic isoform p110α, the second most frequently mutated oncogene and 2) PTEN that encodes the phosphatase and tensin homolog, which is among the most frequently mutated tumour suppressor genes [111].
The importance of PI3K/Akt signaling as a therapeutic target in B cell lymphomas has recently been shown [1, 5, 48-51, 72, 112]. In-vitro studies of lymphoma cell lines and primary cells have revealed PI3K pathway dependence in several lymphoma subtypes. ABC DLBCL cell lines with chronic-active-BCR signaling engage PI3K, which in turn increases anti-apoptotic NF-κB signaling. Recently a group reported a correlation between the deletion of the tumor suppressor PTEN and the activation of the PI3K/Akt pathway in patients and cell lines of the GCB subtype. Selective toxicity with a PI3K inhibitor in PTEN-deficient GCB DLBCL cell lines was observed, suggesting an oncogenic addiction and constitutive activation specific for this subtype [48, 49]. Other mechanisms of dysregulation in the pathway are due to the activity of microRNA clusters that target phosphatase activity involved in the negative regulation of the BCR-PI3K crosstalk, such as miR-17-92(PTEN) and miR-155(SHIP)[72]. BKM-120 (Buparlisib ®, Novartis Pharma AG) is an oral pan-class I PI3K inhibitor that selectively targets all four isoforms of Class I PI3K (α, β, γ, and δ) [113]. BKM-120 has demonstrated antitumour, anti-proliferative and pro-apoptotic activity in several cell lines and xenograft models from cancer [58, 114-119]. Due to the high importance of PI3K/mTOR signaling in cancer and based on genetic screens indicating its role in survival of cancer B-cells, clinical trials of BKM-120 are undergoing in refractory and relapsed Non-Hodgkin Lymphoma [14, 15]. Dual PI3k/mTOR inhibition is particularly effective in blocking AKT activation because it prevents the feedback activation of PI3K signaling normally observed with mTORC1.
inhibitors such as rapamycin [120]. NVP-BEZ235 (BEZ-235 hereafter) is an orally administrated imidazoquinoline derivative compound that acts as a selective dual pan-class I PI3K and mTOR kinase inhibitor, and reversible binds to the ATP-binding cleft of these enzymes inhibiting their catalytic activities [121-124]. Recent studies indicated a potential therapeutic use of BEZ-235 in the treatment of solid cancers, lymphomas and to overcome drug resistance [120, 121, 123-126]. A summary of the alterations of the PI3K pathway in malignant B cells is shown in Figure 9 [72]

![Figure 9. Aberrant BCR-PI3K signaling in malignant cells. Several players of the BCR signaling have been found to be up-regulated by hyper-phosphorylation or over-expression; green indicates increased activity and red abridged activity. This figure is adapted and reprinted from an article published by Pauls et al, 2012 [72]. Copyright permission granted from Frontiers in Immunology.](image-url)
2 Hypothesis and Objectives

Using a panel of GCB DLBCL cells lines, we and others reported that dasatinib has a cytostatic/cytotoxic effect. Dasatinib de novo resistance was associated with SFK independent calcium mobilization upon BCR activation, suggesting differences in BCR signaling between sensitive and resistant cell lines [101, 102]. These results prompted us to select for DLBCL cell lines with acquired resistance to dasatinib and assess for differences in BCR receptor signaling. After chronic exposure and selection using increasing concentrations of dasatinib we obtained stable cell lines that were 30 fold more resistant with respect to parental cell lines. The main objectives of this study were to analyze the phenotypic consequences of chronic exposure to a SFK inhibitor, understand the role of BCR signaling in survival of GCB DLBCL and to shed light on the mechanism involved in acquired resistance to SFK inhibition.

2.1 Hypothesis:

We propose that chronic exposure to SFK inhibition results in the selection of malignant cells able to activate ligand independent BCR signaling through a shift in the regulation of BCR-proximal tyrosine kinase activity.
Objectives:

1. Analyze for differences in pathways important for survival and proliferation such as:
   • BCR signaling
   • PI3K/Akt/mTOR axis

2. Evaluate the cytotoxic effects of compounds targeting the pathways mentioned above.
3 Materials and Methods

3.1 Cell Culture

OCI-Ly1 cell lines were obtained from the Ontario Cancer Institute, Canada. The cells were cultured in RPMI 1640 (Wisent, QC, Canada) supplemented with 10% Fetal bovine serum (Wisent, QC, Canada), 25 mM HEPES (Life Technologies, ON, Canada), 1mM Sodium Pyruvate (Wisent, QC, Canada), 10 U/mL penicillin (Wisent, QC, Canada) 10 µg/mL streptomycin (Wisent, QC, Canada), at 37˚C and 5% CO2 in a humidified incubator. For derivation of dasatinib resistant cells, OCI-Ly1 at passage 7 were selected in progressively increasing concentrations of dasatinib (Cedarlane, Burlington, Canada) or vehicle control. Electroporated cells were cultured in Advanced RPMI 1640 (Life Technologies, ON, Canada) without serum.

3.2 Cytotoxicity Assay

Parental and acquired resistant cell lines were seeded in 96-well plates at a density of 18x10³ cells/well (OCI-Ly1 & OCI-Ly1 B1). The 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cytotoxicity response of the cell lines 72h after treatment with dasatinib (Cedarlane, ON, Canada), PCI-32765 (Ibrutinib) (Selleckchem, TX, US), BKM-120 (Buparlisib) (Selleckchem, TX, US) and NVP-BEZ235 (Dactolisib) (Selleckchem, TX, US). The MTT assay was performed as previously described [127]. Synergy was determined by the formula: a/A + b/B = CI, where a is the IC50 (concentration resulting in 50% of control) of the primary compound
in combination with the secondary compound at concentration b; A is the IC50 of the primary compound without secondary drug; and B is the IC50 of the secondary drug in the absence of primary drug. According to the formula, when CI < 1, the interaction is synergistic, when CI = 1, the interaction is additive, and when CI > 1 there is an antagonistic interaction [127, 128].

3.3 Western Blotting

Cell lines were cultured and treated on 6-wells plates in 3 ml of RPMI 1640, proteins from cell lysates were obtained as previously described[127]. Protein concentration were determined using the Pierce™ Micro BCA protein assay kit( Thermo Fisher, MA, US). 25 ug of protein was resolved on 4-12% polyacrylamide gradient Criterion XT Bis-Tris Precast Gels (Biorad Laboratories, CA, US) and transferred to a nitrocellulose membrane (Biorad Laboratories, CA, US). Membranes were blocked with BSA 5% in Tween 20(0.015%)-TBS for 3 hours at 4 °C and probed overnight with primary antibody at a dilution 1:1000 in T-TBS. The following antibodies were used : rabbit anti-Lyn (Cell Signaling, MA, US), rabbit anti-phospho Lyn Tyr396 (Epitomics, MA, US), rabbit anti-phospho Lyn Tyr 507 (Cell Signaling, MA, US), rabbit anti-phospho SYK Tyr348(Novus, CO, USA), rabbit anti-phospho PLCg2 Tyr759 (Cell Signaling, MA, US), rabbit anti-phospho mTOR Ser2448 (Cell Signaling, MA, US), rabbit anti-mTOR (Cell Signaling, MA, US), rabbit anti-phospho 4EBP-1 Thr37/46 (Cell Signaling, MA, USA), goat anti-β-Actin
(Santa Cruz, TX, US), rabbit anti-CSK (Cell Signaling, MA, US), rabbit anti-phospho AKT Ser473 (Cell Signaling, MA, US), rabbit anti-phospho eIF4E (Cell Signaling, MA, US), BCL2 (Santa Cruz, TX, US), FYN (Santa Cruz, TX, US). Secondary antibodies were horseradish peroxidase conjugated anti-rabbit (GE Healthcare Bio-Sciences, NJ, US), anti-mouse (GE Healthcare Bio-Sciences, NJ, US) or anti-goat (Santa Cruz, TX, US) respectively and were used at a dilution of 1:10000 in T-TBS. Signal was detected using Immobilon Western Chemiluminescent HRP substrate (EMD Millipore, MA, US) and X-ray film (Progene).

3.4 Flow Cytometry- Cell Cycle Assay

For cell cycle analysis, 5x10^5 cells/well were seeded in 6-well plates in 3 ml of RPMI 1640 and grown for 24h or 48h. Upon treatment cells were collected in 15 ml falcon tubes, washed with D-PBS (Wisent, QC, Canada), fixed and permeabilized with 70% ethanol at -20 °C overnight. The next day fixed cells were collected, the ethanol was removed after centrifugation and cells were washed with D-PBS. Then cells were transferred into 1.5 ml tubes and stained overnight with 7-Aminoactinomycin D (BD Biosciences, CA, US) with rotation at 4°C. Next day cells were transferred to flow cytometry tubes and analyzed with the FACSCalibur cytometer (BD Biosciences, CA, US). Results were processed and analysed using the FlowJo v10 platform.
3.5 CRISPR-Cas9 genome editing

In order to target the Lyn kinase and assess its role in BCR signaling the CRISPR-Cas9 genome editing system was used. Expression vectors pSpCas9(BB)-2A-GFP (PX458) and pSpCas9n(BB)-2A-GFP (PX461) designed by the Zhang lab at the Broad institute were purchased from Addgene. Two guides targeting exon 4 of Lyn were designed using the online CRISPR design tool from Zhang Lab[129]. Guides (Table 1) were chosen based on specificity and were purchased from IDT (Integrated DNA Technologies, IA, US).

<table>
<thead>
<tr>
<th>Guide Name</th>
<th>Sequence</th>
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<tr>
<td>Exon4_LYN_Guide1_F</td>
<td>CACCGCCCAGACGACTTGTCTTTCA</td>
</tr>
<tr>
<td>Exon4_LYN_Guide1_R</td>
<td>AACTGAAAGACAAAGTCGTCGAGGCG</td>
</tr>
<tr>
<td>Exon4_LYN_Guide2_F</td>
<td>CACCGGTAGCCTTGTACCCTATGA</td>
</tr>
<tr>
<td>Exon4_LYN_Guide2_R</td>
<td>AAATCTATAGGGGTTACAAGGCTACC</td>
</tr>
</tbody>
</table>

Table 1. Pair of guides targeting Exon 4 of Lyn kinase

Guides were annealed in a thermocycler PTC-100(MJ Research, MA, US) using a temperature cascade from 95 °C to 25 °C. Once annealed the oligos were cloned into the expression vector PX458 (Figure 10). The vector was then chemically transformed with One Shot® Stbl3™ Chemically Competent E. coli cells (Invitrogen). Clones were picked
for overnight liquid culture, plasmid isolation with QIAprep Spin Miniprep Kit (QIAGEN, US) and Sanger sequencing for cloning verification.

Figure 10. Plasmid pSpCas9 (BB)-2A-GFP (PX458)

One day prior to electroporation 4x10⁶ OCI-Ly1 parental and resistant cells per well were seeded in 6-well plates with regular RPMI 1640 in 4 ml final volume. Next day OCI-Ly1 parental and resistant cells were harvested and centrifuged at 1300 rpm for 5 minutes. Cells were then washed with 10 ml of 1X DPBS and centrifuged again for 5 minutes at 1300 rpm. Cells were resuspended with 0.4 ml of advanced RPMI 1640(Life technologies, ON, Canada) and placed in a 0.4 cm pre-chilled cuvette (Biorad, CA, US).
10 ug of plasmid DNA (PX458 with guide) were added into the cuvette and mixed gently. Cells were electroporated using the Biorad Gene Pulser II (Biorad, CA, US) following the manufacturer’s instructions. After electroporation, cells were incubated with serum-free RPMI 1640 at 37 °C, 5% CO2 during 5 h in 6-well plates. After incubation, cells were collected and transferred into a T25 flask with 10 ml of regular RPMI 1640 and were cultured for 72 h. Cells were analyzed for positive GFP expression and were single-cell sorted using the BD FACSARia™ Fusion flow cytometer (BD Biosciences, CA, US) into 96-well plates and incubated for 2 weeks at 37 °C, 5% CO2. Plates were screened for cell viability and cells with normal growth were transferred to 48, 24, and 6 wells plates during 6 weeks. Finally after 8 weeks cells were cultured in T25 flask before western blot analysis and DNA isolation. Lyn knock out analysis through western blot was performed using the rabbit anti-LYN antibody (Cell Signaling, MA, US). Exon 4 of LYN in the isolated clones was PCR amplified and the product was sent to Genome Quebec for sequencing and posterior analysis of the mutation generated by our CRISPR-Cas9 system.

3.6 Statistical Analysis.

All statistical analyses were performed using the SigmaPlot v10 platform (San Jose, CA, USA). Differences between groups were assessed using the paired t-test, t-test, Mann–Whitney test and ANOVA. Differences between groups or associations were considered significant if p<0.05.
4 Results

4.1 B-cell signaling inhibition in GCB DLBCL cell lines.

To assess the effect of drugs in OCI-Ly1, a GCB DLBCL cell line, both parental (Parental hereafter) and OCI-Ly1 cells with acquired resistance to dasatinib (Resistant hereafter), were plated in presence or absence of each compound over 72 hours. The cytotoxicity of the drugs was assessed using the MTT assay. All differences in IC\textsubscript{50} between Resistant lines and Parental counterparts described below were statistically significant with p<0.001. For Western blotting, cells were cultured in the presence of the drugs or vehicle for 24-48 hours, collected and stored at -80C for posterior analysis. The western blots presented in this section are representative of at least three different experiments (N ≥ 3).

4.1.1 Acquired resistance to dasatinib is associated with increased resistance to the BTK inhibitor ibrutinib.

BCR signaling through BTK is regulated by upstream SFKs targeted by dasatinib. To assess for changes in BCR signaling in cells with acquired resistance to dasatinib, we used a clinically relevant non-reversible BTK inhibitor, ibrutinib. Ibrutinib IC\textsubscript{50} of 2.37 +/- 0.09 \(\mu\text{M}\) and 7.79 +/- 0.2 \(\mu\text{M}\) were obtained for Parental and Resistant OCI Ly1 cells respectively. These results suggests that there is cross resistance between dasatinib and ibrutinib in acquired resistance cells. In addition, in Parental but not in Resistant OCI-Ly1 cells, dasatinib sensitized OCI-Ly1 to ibrutinib, the IC\textsubscript{50} obtained with the combination
was: 1.399 +/- 0.195 μM. Results are illustrated in Fig 11 and Fig 12. These results suggest a differential regulation of BTK by SFKs between Parental and Resistant OCI-Ly1 cells.

Figures 11 & 12. Dasatinib and Ibrutinib IC50 in parental (OCI-Ly1) and acquired resistant (OCI-Ly1 R) cell lines. Cross resistance to Ibrutinib in OCI-Ly1 cells.

4.1.2 Acquired resistance to dasatinib results in increased dependency on the mTOR pathway.

SFKs are essential for the recruitment and activation of PI3K in the plasma membrane and it has been reported that tonic BCR signaling channels survival signals through the PI3K/mTOR pathway in mature B-lymphocytes [1, 5, 47, 49]. Therefore, in order to evaluate if chronic SFK inhibition with dasatinib affects PI3K signaling dependency we
use the pan-class I PI3K inhibitor BKM-120 and the dual PI3K/mTOR inhibitor BEZ-235. We did not find a significant difference in BKM-120 sensitivity between Parental and Resistant cells (i.e. IC$_{50}$ values 0.47 +/- 0.03 μM and 0.7 +/- 0.142 μM respectively). In contrast, resistant cells were two fold more sensitive to BEZ-235 than Parental cells with IC$_{50}$s of 0.7 +/- 0.072 μM and 0.34 +/- 0.01 μM respectively. These results suggests that Resistant OCI-Ly1 cells are equally dependent on PI3K than OCI-Ly1 Parental cells but more dependent on mTOR signaling to maintain homeostasis. All differences in IC$_{50}$ between the resistant lines and their parental cell line were statistically significant, p= 0.024. Results are illustrated in Fig 13 and Fig 14.

Figures 13 & 14. BKM and BEZ-235 IC$_{50}$ in parental (OCI-Ly1) and acquired resistant (OCI-Ly1 R) cell lines. Resistant OCI-Ly1 cells are more sensitive to mTOR inhibition.
4.1.3 BKM-120 sensitizes parental and resistant cell lines to Ibrutinib

Based on reports in the literature [1, 5, 27, 47-49, 51, 66, 72, 84] and assuming a contribution of the PI3K pathway to BCR mediated survival in GCB DLBCL, we tested the PI3K inhibitor BKM-120 in combination with ibrutinib. A low concentration (0.1 μM) of each compound in combination with ibrutinib was tested. The difference in IC50 between the treatment with ibrutinib alone versus the combination with the secondary drug was statistically significant, p<0.001. BKM-120 sensitized both parental and resistant cells to ibrutinib and as indicated by the calculated combination index (CI) a synergy between the compounds was observed (CI=0.88 for OCI-Ly1 and CI=0.73 for OCI-Ly1 R). Table 2 and Figure 15 illustrate the results. These results suggest that the contribution of PI3K activation upstream BTK is not affected in the Resistant cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Ibrutinib IC50</th>
<th>Ibrutinib+BKM-120(100nM)</th>
<th>CI</th>
<th>Combination Effect</th>
<th>One way ANOVA</th>
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<tr>
<td>OCI-Ly1</td>
<td>2.37 +/- 0.1</td>
<td>1.6 +/- 0.14</td>
<td>0.88</td>
<td>Synergism</td>
<td>P = &lt;0.001</td>
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<tr>
<td>OCI-Ly1 R</td>
<td>7.79 +/- 0.2</td>
<td>4.6 +/- 0.17</td>
<td>0.73</td>
<td>Synergism</td>
<td>P = &lt;0.001</td>
</tr>
</tbody>
</table>

Table 2. BKM-120 sensitizes parental and resistant cell lines to Ibrutinib
Figure 15. BKM-120 sensitizes parental and resistant cell lines to Ibrutinib. Yet, BEZ-235 sensitizes parental cell line only. BKM-120 [0.1 μM], BEZ-235[0.1 μM]

4.1.4 BEZ-235 sensitized parental cell line OCI-Ly1 to BTK inhibition but has not effect combined with ibrutinib in resistant cells.

The mTOR signaling is one of the aberrantly activated signaling cascades implicated in the pathogenesis of lymphoma[130]. mTOR receives activation signals from different
upstream cascades such as PI3K/Akt and MAPK signaling, therefore is plausible that it can contribute to tonic BCR mediated survival in GCB DLBCL. Moreover, the acquired resistant OCI-Ly1 R cells showed an increased sensitivity to dual PI3K/mTOR inhibition with respect to parental cells. Based on this, a combination treatment between ibrutinib and BEZ-235 was tested. Dual inhibition of PI3K and mTOR sensitized the parental cell line to ibrutinib and a synergy between the compounds was observed (CI=0.61). The difference in IC₅₀ between the treatment with ibrutinib alone versus the combination with the BEZ-235 in the parental cell line was statistically significant, p<0.001., however, the combined treatment of ibrutinib plus BEZ-235 in the acquired resistant cells showed no significant difference in the IC₅₀. Table 3 and figure 15 illustrate the results.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Ibrutinib IC50</th>
<th>Ibrutinib+Bez-235(100nM)</th>
<th>CI</th>
<th>Combination Effect</th>
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<tr>
<td>OCI-Ly1</td>
<td>2.37 +/- 0.1</td>
<td>1.13 +/- 0.02</td>
<td>0.61</td>
<td>Synergism</td>
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<td>OCI-Ly1 R</td>
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<td>7.22 +/- 0.172</td>
<td>1.2</td>
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Table 3. BEZ-235 sensitized parental cell line OCI-Ly1 to BTK inhibition but has not effect combined with ibrutinib in resistant cells.
4.1.5 Dasatinib induced cell cycle arrest in parental but not in resistant OCI-Ly1 cell line.

In order to assess the cytostatic effect of the drug in both parental and resistant cell lines, a cell cycle analysis using flow cytometry was performed. A low concentration (0.1 μM) of dasatinib, which is close to the IC$_{50}$ of the parental cell line and is equivalent to the maximum serum concentrations of dasatinib observed in CML patients taking FDA-approved doses [101] was used and its effect after 48 and 68 h was assessed. A significant cell cycle arrest in G0/G1 in parental cell lines was observed, switching from 50.9 +/- 0.55 to 74.1 +/- 0.8 upon treatment with dasatinib during 48 h. The same pattern was observed after 68 h treatment, switching from 53.1 +/- 0.6 to 67.5 +/- 2.13. All differences between the percentage of cells in each cell cycle phase between the control and dasatinib treatment were statistically significant, p<0.001. The results showed a potent cytostatic effect of dasatinib over the parental cell line OCI-Ly1. Nevertheless, this concentration of dasatinib did not have a cytostatic effect on the resistant cell line after 48 or 68 h of treatment. There were no significant differences in the cell cycle profile between the control (vehicle-treated) and the treated (dasatinib) cells. This result proved that our cells OCI-Ly1 R were resistant to the compound at this concentration. Figures 16, 17, 18, 19, table 4 and 5 illustrate the results. Statistics for 68 h treatment are not shown.
Figure 16. Dasatinib induced cell cycle arrest after 48 h in parental OCI-Ly1 cell line.
Figure 17. Dasatinib induced cell cycle arrest after 68 h in parental OCI-Ly1 cell line.
Figure 18. Dasatinib does not induce cell cycle arrest after 48 h in resistant OCI-Ly1 R cell line.
Figure 19. Dasatinib does not induce cell cycle arrest after 68 h in resistant OCI-Ly1 R cell line.

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<td>28.8</td>
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<td>28.1</td>
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<td>LY1 NT2 48h</td>
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<td>18.4</td>
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<td>SD</td>
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<td>24.6</td>
<td>20.3</td>
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4.1.6 Dasatinib induced activation of BCR downstream targets in acquired resistant OCI-LY1 cells.

With the aim of assessing changes in BCR signaling between parental and resistant cell lines, we tested the effect of a clinically achievable concentration of dasatinib used in the cell cycle analysis (0.1µM) in the phosphorylation steady state of significant targets. Treatment with dasatinib during 24 h showed an important level of inhibition of the signaling (i.e. decrease in phosphorylation and/or expression levels) in parental OCI-LY1 cells, which was expected due to the inhibition of SFKs by dasatinib. In contrast, in resistant cells we observed a significant activation of BCR signaling through the signalosome triggered by the tyrosine kinase LYN using the same treatment. Specifically, dasatinib induced LYN activation as revealed by increased Tyr396 auto phosphorylation together with downstream targets such as SYK(Tyr348) and PLCγ2 (Tyr759), this latter phosphorylated by BTK. In addition, activation of BCR signaling towards PI3K/Akt/mTOR pathway was supported by increased phosphorylation of mTOR (Ser2448) and 4E-BP1 (Thr37/46) after dasatinib treatment. With the same treatment we also observed increased levels of MCL-1, an important antiapoptotic BCL-2 family member regulated by BCR signaling. Figure 20 illustrates the results.
Figure 20. Dasatinib induced activation of BCR downstream targets in acquired resistant OCI-LY1 cells. BCR signaling is assessed through LYN Tyr396 (site of auto phosphorylation and activation), LYN Tyr507 (site of negative regulation phosphorylated survival and proliferation). 

Das: Dasatinib [0.1 μM]-24h

NT: non-treated
by CSK), SYK Tyr348 (site of auto-phosphorylation and activation), PLCγ2 Tyr759 (site phosphorylated by BTK). PI3K/mTOR was assessed through: mTOR Ser2448 (site of activation) and 4E-BP1 Thr37/46 (site of phosphorylation that results in the release of eIF4E and posterior increase in cap-translation).

4.1.7 Design and generation of two new constructs targeting exon 4 of lyn.

Since LYN is involved in proximal BCR signaling and, as shown by our results with resistant cells, is activated in its kinase domain by dasatinib, we reasoned that dasatinib resistance is due in part to changes in regulatory networks downstream of LYN and BCR. As such, to more fully elucidate the role of LYN in de novo and acquired resistance to dasatinib, we decided to knock out the kinase with CRISPR-Cas9 technology and evaluate the phenotypic and pharmacological consequences in both parental and acquired resistant cell lines. In order to carry out experiments using CRISPR-Cas9 technology, all-in-one DNA CRISPR-Cas9 vectors targeting the lyn proto-oncogene were generated. Two pairs of guides were designed using the online CRISPR tool from the Zhang group at the Broad institute [129]. Guides were annealed and cloned into the backbone expression vector PX458 and new vectors were chemically transformed with competent cells. Clones were picked for overnight liquid culture, plasmid isolation and sequencing for cloning verification. Two new constructs PX-458 G1 and PX-458 G2 harboring our lyn-exon 4 guide scaffolds were generated. Figure 21 illustrates the results.
Figure 21. Generation of constructs PX-458 G1 (A) and PX-458 G2 (B) targeting exon 4 of *lyn*.
4.1.8 Generation of *lyn* knockout cell lines derived from parental OCI-Ly1 cell line and a *lyn* mutant cell line derived from resistant cell line OCI-Ly1 R through the use of CRISPR-Cas9 genome edition.

Once the validation of the CRISPR-Cas9 vectors PX458-G1 and PX458-G2 was confirmed through PCR and Sanger sequencing, both parental and resistant cells were electroporated with the expression vectors. After 72 hours of incubation in cell culture, single cells were sorted in 96-well plates using fluorescence-activated single cell sorting. Three *lyn* knockout monoclonal stable cell lines (E11, E8 and E20) derived from the parental cell line OCI-Ly1 and one *lyn* mutant monoclonal cell line (E5) derived from the resistant cell line OCI-Ly1 R were generated.

Genome edition of *lyn* in exon 4 was verified by Sanger sequencing and protein deficiency through western blot analysis. Knockout of *lyn* was not lethal in our parental cells, suggesting that other SFKs can compensate its role in our GCB DLBCL cell lines. Preliminary characterization of the mutation in clone E11 showed insertion and deletions in exon 4 mediated by CRISPR-Cas9 and the non-homologous end joining repair system. Genotyping was performed through Sanger sequencing and sequence alignment. Figure 22 and 23 illustrated the results.
Figure 22. Generation of three \textit{lyn} knockout cell lines (E11-E8-E20) derived from parental OCI-Ly1 cell line and a \textit{lyn} mutant cell line (E5) derived from resistant cell line OCI-Ly1 R
Figure 23. DNA sequencing chromatogram showing mutations in E11 clone, the CRISPR-Cas9 genome edition resulted in two deletions and one insertion in exon 4 of *lyn*. 
4.1.9 *lyn* knockout sensitized OCI-Ly1 cell line to BTK inhibition.

Although it seems that there is a redundancy in the role of SFKs in BCR signaling, literature suggest that among the SFKs involved in this pathway LYN has a preponderant role as a signaling modulator [77, 81, 83, 131, 132]. In order to evaluate changes in the sensitivity to BTK inhibition due to LYN deficiency, we tested knockout cells lines E11, E8 and E20 using ibrutinib. We found that the three LYN -/- cell lines (E11, E8 & E20) were sensitized to ibrutinib, with the IC$_{50}$ switching from 2.37 +/- 0.1 µM (parental OCI-Ly1 cell line) to 0.64 +/- 0.06 µM (E11), 0.5 +/- 0.03 µM (E20) and 0.49 +/- 0.01 µM (E8).

The sensitivity results obtained in the *lyn* knockout cell lines were in accordance with our previous pharmacological results showing a sensitization with the combination of ibrutinib and dasatinib-mediated LYN inhibition. Altogether, our results showed a direct involvement of the LYN kinase in resistance to BTK inhibition in parental OCI-Ly1 cells. All difference in the IC$_{50}$ between the parental cell line OCI-Ly1 and the Lyn -/- knockout cell lines(E11-E8-E20) were statistically significant, p<0.001. Figure 24 illustrates the results.
**Figure 24. lyn knockout sensitized OCI-Ly1 cell line to BTK inhibition.**

4.1.10 *lyn* knockout sensitized OCI-Ly1 cell line to SFK inhibition.

Despite the fact that Dasatinib targets several SFKs in B lymphocytes, reports suggest that its antiproliferative effect in DLBCL is prominently based on LYN inhibition [101, 102]. Hence, to assess changes in the sensitivity to SFK inhibition due to LYN deficiency we tested our knockout cell line E11 against dasatinib treatment. We found that knockout cell line E11 was sensitized to dasatinib, with the IC$_{50}$ switching from 0.11 +/-0.01 μM (OCI-Ly1 cell line) to 0.004 +/- 0 μM (E11). The difference in the IC$_{50}$ between the parental
cell line (OCI-Ly1) and the Lyn -/- knockout cell line (E11) was statistically significant, p<0.001. Figure 25 illustrates the results.

![IC50 Dasatinib](image)

**Figure 25. lyn knockout sensitized OCI-Ly1 parental cell line to SFK inhibition.**

4.1.11 Lyn mutant resistant cell line OCI-Ly1 R (E5) is more sensitive to SFK inhibition, yet resistant to BTK inhibition.

LYN activity is not only regulated by its phosphorylation status in the kinase domain, but also by its interactions through its SH2 and SH3 domains[81]. These domains are involved in the generation of the inactive configuration of the kinase and disruptions on these domains lead to deregulated activation of the enzyme. Moreover, the SH3 domain
is also important for interaction with substrates with proline rich regions (PXXP consensus) and is also involved in the localization of the kinase in specific cellular compartments [79, 81, 133-135].

Based on our previous results showing dasatinib-induced activation of BCR downstream targets in acquired resistant OCI-LY1 R cells and the fact that that our lyn mutant resistant cells (E5) harbor a heterozygous mutation in exon 4 of Lyn affecting the SH3 domain, we decided to tested these cells against dasatinib and ibrutinib treatment respectively.

Paradoxically, contrasting results between the two drugs were obtained. The acquired resistant cells were sensitized to dasatinib with an IC\textsubscript{50} switching from 3.39 +/- 0.3 μM (OCI-Ly1 R) to 0.72 +/- 0.04 μM (E5), a 4.7-fold decrease in sensitivity to the compound. In the other hand, the cells showed an increase in the resistance to BTK inhibition through ibrutinib treatment, with an IC\textsubscript{50} switching from 7.79 +/- 0.23 (OCI-Ly1 R) to 9.53 +/- 0.08 μM (E5) respectively. All difference in the IC\textsubscript{50} for both drugs between the acquired resistant cell line (OCI- Ly1 R) and the derived Lyn mutant (E5) were statistically significant, p<0.001. Figure 26 and 27 illustrate the results.
Figure 26. *lyn* mutant resistant cell line E5 showed restoration of SFK inhibition sensitivity with dasatinib, yet they are more resistant to BTK inhibition.

Figure 27. *lyn* mutant resistant cell line E5 is more resistant to BTK inhibition.
4.1.12 PI3K inhibition through BKM-120 treatment is not affected due to LYN deficiency, however resistance against dual inhibition of PI3K and mTOR signaling is increased in lyn mutant resistant cells E5.

Considering a potential unique role for LYN in the recruitment of PI3K to cell membrane in our parental and resistant cell lines, we evaluated the effects of lyn knockout in PI3K/mTOR signaling in the cell line E11 with the PI3K inhibitor BKM-120 and the dual inhibitor BEZ-235. No difference in the sensitivity against the two compounds between the parental cell line OCI-Ly1 and the derivative knockout cell line E11 was found.

The IC\textsubscript{50} changes from 0.466 +/- 0.03 \mu M (OCI-Ly1) to 0.5 +/- 0.09 \mu M (E11) for BKM-120 and 0.7 +/- 0.072 \mu M (OCI-Ly1) to 0.83 +/- 0.03 \mu M (E11) for BEZ-235 were not statistically significant. However, when we tested the lyn mutant OCI-Ly1 cell line (E5) against BEZ-235 treatment, we found an important increase in the IC\textsubscript{50}: from 0.34 +/- 0.01 \mu M (OCI-Ly1 R) to 1.65 +/- 0.12 \mu M (E5), suggesting a potential oncogenic addiction in these cells. The difference between the IC\textsubscript{50} obtained for the acquired resistant cell and the derived E5 resistant cell was statistically significant, \( p = 0.007 \). Figure 28 and 29 illustrate the results. These results suggest that the sensitivity to BEZ-235 treatment in the E5 cells could be mediated by the mutant LYN kinase.
Figures 28 and 29. PI3K inhibition through BKM-120 treatment is not affected due to LYN deficiency, however resistance against dual inhibition of PI3K and mTOR signaling is increased in \textit{lyn} mutant resistant cells E5.

4.1.13 Increased activation of SYK kinase and phospholipase C gamma-2 (PLC\gamma2) in \textit{lyn} knockout cell lines E11, E20 and E8 is observed.

Aiming to assess changes in BCR signaling, specifically the cascade SFK-SYK- PLC\gamma2, related to the absence of the LYN kinase, we did a western blot analysis after 24 h of cell culture and found that our three \textit{lyn} knockout cell lines presented an increased phosphorylation in SYK (Tyr 348) and PLC\gamma2 (Tyr759) with respect to the basal level of the protein in the parental cell line OCI-Ly1. Both SYK and PLC\gamma2 are key players in the signalosome triggered by the Src family kinases upon ITAM phosphorylation in the B-cell receptor complex. Additionally, aiming to evaluate BCR activation by other Src-family
kinases besides LYN we assessed the expression of the FYN kinase in our knockout clones and no difference in protein levels between the parental and LYN knockout clones was observed. This results suggested that SYK kinase activity is independent of LYN and may be compensated by other Src kinases such as FYN. Figure 30 illustrates the results.

Figure 30. Increased activation of SYK Tyr348 and phospholipase C gamma-2 (PLCγ2) Tyr759 in lyn knockout cell lines E11, E20 and E8 is observed. FYN kinase expression is not altered in cell lines E11, E20 and E8.

4.1.14 lyn mutant E5 displays increased activation of AKT Ser473

AKT plays a crucial role in the PI3K/Akt/mTOR pathway and it has been reported to be involved in DLBCL [136]. In order to evaluate AKT activity we did a western blot and found that the lyn mutant cell line E5 displays increased phosphorylation of AKT at Ser473, this residue is phosphorylated by mTOR in a rapamycin-insensitive complex with rictor.
and Sin1[137, 138]. However, we did not observe differences in mTOR phosphorylation between the resistant OCI-Ly1 R and the lyn mutant E5 cell line at Ser2448, suggesting that this upregulation of AKT could be mediated by other mechanisms. Figure 31 illustrates the results.

![Figure 31](image)

Figure 31. lyn mutant E5 displays increased activation of AKT Ser473, yet phosphorylation of mTOR Ser2448 is not altered.

4.1.15 lyn knockout cell lines E11, E8 and E20 showed an increase in the phosphorylation of mTOR downstream targets such as 4E-BP1 and eIF4E.

The mTOR pathway is involved in proliferation due to modulation in mRNA translation. In order to assessed BCR-downstream distal targets through mTOR signaling we evaluated 4E-binding protein 1(4E-BP1) and the eukaryotic initiation factor 4E (eIF4E) in our CRISPR cell lines. We observed that our edited cell lines with lyn knockout presented an increase in the phosphorylation of 4E-BP1 at Thr37/46 and eIF4E at Ser209, with a
particular important up regulation in the E8 cell line. This results suggested that these knockout cells could have an increase in cap-dependent translation. Figure 32 illustrates the results.

Figure 32. lyn knockout cell lines E11, E8 and E20 display an increase in the phosphorylation levels of mTOR downstream targets eIF4E Ser209 and 4EBP-1 Thr37/46.
4.1.16 *lyn* mutant E5 cell line showed increased activity of Lyn at Tyr396 but this is no longer increased upon dasatinib treatment.

Previously we showed a dasatinib-induced activation of BCR downstream targets in acquired resistant OCI-LY1 cells, however this activation is no longer observed in *lyn* mutant E5 cells. Although, at basal level they already presented an increase in the levels of phosphorylation of LYN Tyr396 with respect to the resistant cell line OCI-Ly1 R, nonetheless, this phosphorylation is not altered upon treatment with dasatinib 0.1 μM.

Moreover, phosphorylation at LYN Tyr507 is not affected in E5 clone and still is down regulated by dasatinib. Phosphorylation at the kinase domain is critical for the correct functioning of the protein, however, the SH3 domains also play a crucial role in the conformation stability of the kinase and the interaction of the kinase with its targets [139]. Moreover, the docking of the kinase through the SH3 and SH2 domains could be impaired in *lyn* mutant E5 cells and this could be affecting the kinase activity in the cell membrane or the cytosol. Phosphorylation at the negative regulatory residue LYN Tyr507 is controlled by the C-Src kinase (CSK) which happens to also be a target of dasatinib. Therefore dasatinib inhibits the inhibitor of LYN and this has not been altered in the E5 clone. Figure 33 illustrate the results.
Figure 33. Dasatinib does not induce Lyn Tyr396 activation in lyn mutant cell line E5, yet inhibitory residue at Tyr507 is still affected by the compound.

4.1.17 lyn mutant E5 cell line showed no change in proximal and distal BCR downstream targets upon dasatinib treatment.

In order to evaluate the effects of dasatinib at 0.1 µM after 24 hours treatment a western blot analysis was performed. No changes in phosphorylation of downstream targets of BCR signaling such as SYK (Tyr 348) and PLCγ2 (Tyr759) or distal targets such as mTOR Ser2448, 4E-BP1 (Thr37/46) or eIF4E (Ser209) was observed in E5 clone upon treatment. These results suggest that although LYN Tyr396 activity it seems to be upregulated in the E5 clone, this signal is not transduced in the pathway and activity of downstream players is not affected. Figure 34 illustrates the results.
OCI-Ly1 R
E5(LYN mutant)

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LYN
LYN (Tyr396)
LYN (Tyr507)
SYK(Tyr348)
PLCγ2(Tyr759)
mTOR(Ser2448)
4EBP-1(Thr37/46)
eIF4E(Ser209)
BCL-2
β-Actin

NT : Non-treated
Das 100nM : Dasatinib [0.1 μM]-24H
Das IC10 : Dasatinib [0.009 μM]-24H

Figure 34. Dasatinib at 0.1 μM does not induce BCR signaling in E5 clone.
5 Discussion and Conclusion

In the molecular era of rituximab-based first-line therapy, the next breakthrough in the treatment of DLBCL will be the identification of driver mutations and the design of personalized therapies using combinations of agents targeting pathway addictions responsible for the survival of specific DLBCL subtypes and drug resistance. B-cell receptor (BCR) signaling has emerged as an important oncogenic pathway which promotes growth and survival in several lymphoma subtypes [1, 5, 31, 140]. The initiation and amplification of BCR signaling is determined by the efficiency of the recruitment and activation of the Src, BTK and SYK family kinases to the BCR complex. Recent publications and results of clinical trials show the importance of targeting components of proximal and distal BCR (LYN, SYK, BTK and PI3K) signaling with tyrosine kinase inhibitors in DLBCL [1, 3-5, 26, 27, 31, 47-49, 55, 72, 90, 92, 93, 96, 101, 102, 104, 108, 110, 139-145]. Both highly specific (Ibrutinib) and pan-inhibitors (Dasatinib) are useful tools for the study of BCR signaling and its role in the cancer biology of DLBCL. In the current study, we investigated the effects of SFK, BTK and PI3K/mTOR inhibition in the GCB DLBCL cell line OCI-Ly1. This cell line harbors a p53 mutation, BCL2 and MYC translocations and therefore represents a subtype of DLBCL with extremely poor prognosis. Consequently, there is a need to explore new potential treatments for this genetic background. Preliminary results in the laboratory and previous publications [101,
showed that dasatinib has a cytostatic/cytotoxic effect in GCB DLBCL cell lines. We obtained a stable acquired resistant cell line (OCI-Ly1 R) by exposing sensitive cells continuously to increasing drug concentrations and selecting surviving clones. The main objectives of this study were to analyze the phenotypic consequences of this chronic exposure to dasatinib, understand the role of BCR signaling in survival of GCB DLBCL and to shed light on the mechanism involved in the acquired resistance to SFK inhibition. Two previous studies suggested SFK-independent activity of SYK, constitutive activation of LYN and AKT activity as potential mechanisms of resistance to dasatinib [101, 146].

We found that cells with acquired resistance to dasatinib displayed cross resistance to the BTK inhibitor ibrutinib, this is consistent with results found in the literature [1, 26, 140] that indicate BTK in BCR signaling is regulated by upstream Src-family kinases such as LYN, FYN and HCK, all of which are targeted by dasatinib. However, when we combined ibrutinib with fixed concentrations of dasatinib, we found sensitization to ibrutinib in parental cells but not in acquired resistant cells. This result suggests that the regulation of BTK through SFK activity is different between parental and resistant cells. Young et al, [1] proposed a BCR signaling cascade model where BTK is regulated by SYK and BLNK which are activated by LYN, this model suggests a SFK-dependent BTK activity which could be targeted by ibrutinib. In addition, our results show that PI3K signaling contributes to ibrutinib resistance and proliferation in both parental and acquired resistant cells, with no significant difference in the drug response between
them. This data is in agreement with several studies [47-49, 72, 140] that suggest an involvement of PI3K signaling in survival of GCB DLBCL. Moreover, it has been reported that tonic BCR signaling channels survival signals only through PI3K in mature B cells [66].

Furthermore, when we tested the combination of ibrutinib and the PI3K inhibitor (BKM-120). A synergy between the compounds was found and both parental and resistant cells were sensitized to ibrutinib. These results confirm the contribution of PI3K in the survival of GCB DLBCL and also that PI3K activity upstream BTK is not affected in resistant cells. On the other hand, when we tested the dual inhibitor BEZ-235, which targets both PI3K and mTOR, we found that acquired resistant cells were more sensitive to this compound. This result and the cytotoxicity response found with BKM-120 suggest that acquired resistant cells were equally dependent on PI3K when compared to parental cells but more dependent on mTOR to maintain homeostasis. When ibrutinib was combined with BEZ-235 we found sensitization on parental but no significant response in acquired resistant cells. A possible explanation for this contrasting result is that the simultaneous targeting of the highly important BTK-PI3K and mTOR pathway results in the activation of compensatory survival pathways such as chemokine or integrin signaling in resistant cells. It is important to mention that Seda et al. [27] reported that ibrutinib treatment leads to AKT upregulation, which could explain a contrasting effect between BCR inhibition(ibrutinib) and PI3K/mTOR inhibition (BEZ-235). It would be interesting to
investigate the chemokine and integrin signaling profiles of these cells when treated with inhibitors that affect BCR signaling. Furthermore, it would be interesting to analyze BCR and PI3K/mTOR signaling in these cells using synergistic combinations of ibrutinib and BKM-120/BEZ-235. These experiments will help correlate our cytotoxicity data with biochemical profiles and enhance understanding of potential cross talk between BCR and other pathways that are important for survival and proliferation of cancerous B lymphocytes.

Although chronic BCR signaling is a hallmark of the ABC subtype and a critical role of tonic BCR has been reported in Burkitt Lymphoma [147, 148], the role of tonic BCR signaling in GCB DLBCL needs to be clarified and requires further research to shed light on the mechanisms involved.

In this regard, our results suggest an important role of tonic BCR signaling in proliferation and survival of the GCB DLBCL cell line OCI-Ly1. This is supported by western blot analysis showing ligand-independent tyrosine kinase activity and also by the response of cells to treatments targeting BCR and PI3K/mTOR signaling. Moreover, both parental and acquired resistant cells showed a regulation of signaling mediated by LYN kinase, a master modulator of tonic, chronic and normal BCR signaling [1, 77, 81, 83, 140]. Results with the parental cell line OCI-Ly1 showed that LYN contributes to Dasatinib and Ibrutinib resistance. Furthermore, lyn knockout experiments on this cell line showed that other Src-
family kinases can compensate LYN's role in survival and the lack of the kinase is not lethal in these cells.

In addition, the *lyn* knockout cell line E11 was sensitized to dasatinib and ibrutinib treatment, suggesting a unique role for LYN in the activation of BTK. This also indicates the possibility of another potential mechanism of survival that is LYN-dependent and it may not be related with BCR signaling. Is important to highlight that the sensitization to ibrutinib was found in three different *lyn* knockout clones: E11, E8 and E20, which were independently selected using single-cell-sorting and CRISPR-Cas9 genome editing, showing that there is a significant correlation between *lyn* expression/activity and ibrutinib resistance.

In addition, western blot and cytotoxicity results with our lyn knockout cells showed LYN's role in activation and inhibition of the pathway. Our data demonstrated that LYN has a key unique role in the negative regulation of SYK and PLCγ2 that cannot be compensated by the remaining SFKs. This is consistent with literature suggesting a unique role for LYN in the phosphorylation of a negative residue in SYK [26, 77] and its role as the key negative modulator of BCR signaling [1, 26, 77, 79, 81, 131, 132, 140]. These results also suggest that SYK kinase activity may be independent of LYN and may be either compensated by other Src-family kinases such as FYN or HCK. Nevertheless, we cannot ruled out the possibility of an autonomous activation of SYK as previously reported [94, 101].
Regarding the mechanism underlying acquired resistance to dasatinib in our resistant cells, our western blot results suggest a pivotal role for LYN in promoting survival of these cells. Although at basal levels the resistant cells display low tyrosine kinase activity (LYN, SYK and PLCg2) this kinase cascade is activated upon treatment with dasatinib and the signal is transduced all the way down to mTOR and 4E-BP1. Since both of these proteins are reported to be involved in survival and proliferation, this signaling rewiring could explain the mechanism of acquired resistance in these cells.

LYN's unique role in the negative regulation of BCR signaling and its ability to phosphorylate and activate immunoreceptor tyrosine-based inhibition motifs (ITIMs) which allow the recruitment of phosphatases involved in negative regulation of the signaling, makes this kinase an important modulator of BCR signaling. In addition, LYN is important for the recruitment of CSK into lipids rafts of the plasma membrane through tyrosine phosphorylation of Cbp/PAG. Recruited CSK downregulates Src-kinase activity (including LYN) by phosphorylation of the residue Tyr507, resulting in down regulation of signaling [81]. The drug addiction of the resistant cells acquired during the chronic exposure to dasatinib could be explained by this dual role of LYN in BCR signaling, and the interactions of the kinase with positive and negative regulators such as SHP-1, SHIP-1 (recruited within the ITIMs), CD45 and CSK. It is important to mention that dasatinib not only targets Src-family kinases involved in BCR, but also CSK, resulting in a downregulation of this negative regulator. By targeting negative regulators of the Src
kinase signaling pathway, dasatinib in a sense is promoting Src kinase activity by blocking the activity of inhibitory kinases. We have assessed the protein profile of CSK and SHP-1 and found no differences between parental and resistant cells. However, it would be interesting to investigate levels of expression and activity of CD45, a positive effector of BCR signaling and evaluate its role in the regulation of LYN activity. Based on our results, it seems that LYN is responsible for the activation and rewiring of signaling in acquired resistant cells, however a knockout experiment will be necessary to confirm this hypothesis.

Although the resistant clone E5 still displays LYN kinase activity, which is upregulated at LYN Tyr396, this mutant cell line was not able to activate the pathway upon dasatinib treatment like the acquired resistant OCI-Ly1 R from which it was derived. This may be due to the mutation this cell line harbors in the SH3 domain, which is critical for the interaction between the kinase and its substrates [139]. Although, the LYN mutant E5 clone was sensitized to dasatinib, it turned out to be more resistant to ibrutinib and BEZ-235. This could be explained by the increase in phosphorylation of Akt Ser473 that these cells display. The kinase Akt is not only involved in mTOR signaling but it also interacts with multiple downstream targets important for survival, proliferation, angiogenesis, growth and metabolism [149].
Altogether, three different LYN responses were found upon dasatinib treatment which indicates the amazing plasticity cancer cells have to respond against anti-cancer agents, furthermore, our results support the role of LYN in the survival of germinal center B-cell like DLBCL cells and further our understanding of this important modulator of BCR signaling.

Our study demonstrate the applicability of CRISPR-Cas9 technology in genome editing of cancer cell lines, specifically B lymphocytes, cells well known to be difficult to transfect and manipulate genetically. It is important to mention that our lyn knockout cell lines generated are important models to study the role of this kinase in BCR signaling and other tyrosine kinase receptors important for survival in DLBCL.

In summary, our results show that tonic BCR signaling is important for the survival of GCB DLBCL and that LYN kinase, although not essential for survival, plays a pivotal role in the modulation of this pathway. Dasatinib and ibrutinib are powerful tools for the study of BCR signaling and the understanding of the mechanism involved in this pathway, moreover, due to its recent approval by the FDA and the ongoing clinical trials in DLBCL, is important to continue doing research with ibrutinib to test its effect in different genetic backgrounds and to try to elucidate potential mechanisms of resistance. Is important to mention that Cheng et al [110] recently showed that dasatinib could overcome ibrutinib resistance in CLL patients, this indicates the importance of pan-inhibitors vs highly specific inhibitors such as ibrutinib. Further design of inhibitors targeting SFKs with more
specificity than dasatinib could open an important therapeutic window to target BCR signaling and could help in the treatment of patients who do not respond well to R-CHOP or ibrutinib. The synergy found between BTK inhibition and PI3K/mTOR inhibition in parental cells shows the importance of targeting multiple pathways in DLBCL and also supports the importance of tonic BCR signaling and PI3K in the GCB background. Further research is necessary to clarify the activation of Src-family kinases in tonic BCR and its cross talk with PI3K signaling. Drug resistance unfortunately will continue to represent a challenge in the treatment of DLBCL, as such, research on this field is important for the improvement in lymphoma treatment. Finally, the application of genomic and functional screenings should help researchers and physicians have a better understanding of lymphoma biology and the development of combination therapeutics that will improve the treatment of DLBCL through personalized medicine.
6 References


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