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IN VITRO AND *IN VIVO* STUDIES OF BRUTON TYROSINE KINASE (BTK) MUTATIONS & INHIBITION

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In vitro and *in vivo* studies of Bruton tyrosine kinase (BTK) mutations & inhibition

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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…to those who inspired me!

ABSTRACT

Bruton tyrosine kinase (BTK) is a non-receptor protein kinase that belongs to the TEC family kinases. It plays an important role in the B-cell receptor signaling pathway (BCR) and its pharmacological inhibition has been demonstrated as an effective strategy for the treatment of B-cell malignancies. Ibrutinib, acalabrutinib and zanubrutinib are small molecules and irreversible BTK binders that have been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of several B-cell malignancies. Irreversible inhibitors block BTK catalytic activity by covalently binding to the cysteine (C) 481 located in the kinase domain. Mutations at this residue abrogate the possibility of forming a covalent bond, thereby decreasing the efficacy of the inhibitor. The most common mutation found in treated patients is the cysteine-481 to serine substitution (C481S). However, other less frequent substitutions have also been identified, such as, T474I and T474S substitutions in the BTK gatekeeper residue or PLCγ2 gain-of-function substitutions e.g. S707Y and R665W.

In **paper I** we studied a novel C481S knock-in mouse model. Our analysis of these mice reveled no phenotype alterations, as compared to wild-type mice, and demonstrated that C481S substitution has no detectable effect on BTK´s function or on the development of hematopoietic cells. We demonstrated that isolated B-lymphocytes carrying C481S were resistant to irreversible but sensitive to reversible BTK inhibitors (BTKis). This was achieved by analyzing BTK catalytic activity, cell-viability and expression of cell activation markers. Additionally, we confirmed that irreversible BTKis impaired T-lymphocyte activation in a BTK independent manner. This demonstrates the potential of this mouse model to be used in the study of BTK-independent, both therapeutic and adverse, effects caused by irreversible BTKis.

Resistance to BTKis has become one of the most critical concerns in long term ibrutinib treated patients. The cause of the resistance to irreversible BTKis is less frequently associated to the gatekeeper residue, in contrast what is observed for other kinase inhibitors such as the fusion-protein BCR-ABL inhibitor imatininb or the EGFR inhibitor gefitinib. In **paper II** we aimed to understand the role of gatekeeper and combined gatekeeper/C481 BTK variants in the resistance to reversible and irreversible BTKis. We evaluated protein expression, catalytic activity and susceptibility to BTKis of 16 BTK single and double variants. We found that double T474I/C481S, T474M/C481S and T474M/C481T variants were insensitive to ≥16 fold irreversible inhibitor pharmacological serum concentration. On the other hand, reversible BTKis showed a variable inhibition pattern. RN486 seemed to have highest therapeutic potential for patients that develop resistance to combined gatekeeper/C481 BTK variants.

LIST OF SCIENTIFIC PAPERS

I. H. Yesid Estupiñán, Thibault Bouderlique, Chenfei He, Anna Berglöf, Dhanu Gupta, Osama Saher, Miguel Ángel Daza Cruz, Lucia Peña-Perez, Liang Yu, Rula Zain, Mikael C. I. Karlsson, Robert Månsson* and C. I. Edvard Smith* **Novel mouse model resistant to irreversible BTK inhibitors: a tool identifying new therapeutic targets and side effects.** *Blood Adv* 2020; 4 (11): 2439-2450.

II. H. Yesid Estupiñán, Qing Wang, Anna Berglöf, Gerard C. P. Schaafsma, Yuye Shi, Litao Zhou, Dara K. Mohammad, Liang Yu, Mauno Vihinen, Rula Zain and C. I. Edvard Smith. **BTK gatekeeper residue variation combined with cysteine 481 substitution causes super-resistance to irreversible inhibitors acalabrutinib, ibrutinib and zanubrutinib.** *Leukemia* 2021; doi: 10.1038/s41375-021-01123-6

Additional scientific publications and manuscripts not included in the thesis.

I. H. Yesid Estupiñán, Anna Berglöf, Rula Zain and C. I. Edvard Smith. **Comparative analysis of BTK inhibitors and mechanism underlying adverse effects**. *Front. Cell Dev. Biol* 2021; doi: 10.3389/fcell.2021.630942

II. Tom A. Mulder, Lucía Peña-Pérez, Anna Berglöf, Stephan Meinke, H. Yesid Estupiñán, Kia Heimersson, Rula Zain, Robert Månsson, C. I. Edvard Smith* and Marzia Palma* **Ibrutinib has time-dependent on- and off-target effects on plasma biomarkers and immune cells in chronic lymphocytic leukemia.** *HemaSphere* 2021. (Accepted for publication)

III. Qing Wang, Anna Berglöf, A. Charlotta Asplund, Rula Zain, Igor Resnick, H. Yesid Estupiñán, Sofia Khan, Mauno Vihinen, Gerard C. P. Schaafsma and C.I. Edvard Smith. **Acquired BTK mutations suggest tumor suppressor activity in leukemia and lymphoma subsets.** (Manuscript)

IV. H. Yesid Estupiñán, Thibault Bouderlique, Chenfei He, Anna Berglöf, Dhanu Gupta, Liang Yu, Rula Zain, Mikael C.I. Karlsson, Robert Månsson and C.I. Edvard Smith. **BTK tyrosine 223 phosphorylation marks activation but is functionally dispensable.** (Manuscript)

V. Burcu Bestas, Qing Wang, H. Yesid Estupiñán, Dimitri A. Stetsenko, Christian J. Leumann, Matthew J.A. Wood, Michael J. Gait, Dara K. Mohammad, Thibault Bouderlique, Dhanu Gupta, Oscar OE Wiklander, Shabnam Kharazi, Chenfei He, Robert Månsson, Mikael C. I. Karlsson, Taavi Lehto, Karin E. Lundin, Anna Berglöf, Rula Zain and C. I. Edvard Smith**. Chemically modified splice-switching oligomers rescue phenotype in Btk/Tec double deficient mouse model of X-linked agammaglobulinemia.** (Manuscript)

*Authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

AF	Atrial fibrillation
AID	Activation-induced cytidine deaminase
ALK	Anaplastic lymphoma kinase
ARA	Autosomal recessive agammaglobulinemia
ATP	Adenosine triphosphate
BCR	B-cell receptor
BCR-ABL	Breakpoint cluster region / Tyrosine kinase ABL1
BLK	B-lymphoid kinase
BM	Bone marrow
BMX	Bone marrow-expressed kinase
BTK	Bruton tyrosine kinase
BTKis	BTK inhibitors
CD	Cluster of differentiation
cGVHD	Chronic graft-versus-host disease
CLL	Chronic lymphocytic leukemia
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ERBB2/HER2	Human epidermal growth factor receptor 2
ERBB4/HER4	Human epidermal growth factor receptor 4
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FMO	Fluorescence minus one
HSCs	Hematopoietic stem cells
Ig	Immunoglobulin
IL	Interleukin
IP3	Inositol-1,4,5-trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITK	Inducible T-cell kinase
JAK3	Janus kinase 3
KO	Knock-out
LPS	Lipopolysaccharide
MCL	Mantle cell lymphoma
MES SDS	4-morpholineethane sulfonic acid - sodium dodecyl sulfate buffer
MZ	Marginal zone
MZL	Marginal zone lymphoma

NMPA	National Medical Products Administration
ORR	Overall response rate
PBS	Phosphate buffered saline
PEI	Polyethylenimine
PH	*Pleckstrin homology* domain
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PLCγ2	Phospholipase C-γ2
PMDA	Pharmaceuticals and Medical Devices Agency
PTK	Protein tyrosine kinase
RIPA	Radioimmunoprecipitation assay buffer
RLK/TXK	Resting lymphocyte kinase/ T and X cell expressed kinase
RNA	Ribonucleic acid
R/R	Relapsed/refractory
SH	*SRC homology* domain
SLL	Small lymphocytic leukemia
SYK	Spleen tyrosine kinase
TEC	Tyrosine kinase expressed in hepatocellular carcinoma
TFKs	TEC family kinases
TH	*Tec homology* domain
TNFα	Tumor necrosis factor α
WM	Waldenström macroglobulinemia
XLA	X-linked agammaglobulinemia

1 INTRODUCTION

1.1 B-lymphocyte development

After birth and continuously throughout the whole life, B-lymphocytes develop from hematopoietic stem cells (HSCs) in the bone marrow (BM). The first stage of the specific B-cell development starts with the pro-B cell, which expresses the B-cell lineage-specific cluster of differentiation (CD) 19. It is at this stage of the development that the immunoglobulin (Ig) heavy chain initiates the diversity (D) and joining (J) rearrangement (Boekel et al., 1995). The transition from pro-B cell to the next stage pre-B cell, depends on a productive variable (V) DJ rearrangement and the expression of the μ heavy chain associated with the surrogate light chain (Übelhart et al., 2015). Pre-B cell loses CD117 (c-kit) marker, gains CD25 expression and undergoes VJ light chain rearrangement. Efficient heavy-light chains pairing and the consequent expression of the formed and functional B-cell receptor (BCR) are required for the transition from pre-B to immature B-cell stage. Immature B-cells that will exit from the BM migrate to the second lymphoid tissues and upon alternative splicing acquire the IgD surface marker, which in co-expression with IgM characterizes transitional and mature B-cells populations (Osmond, 1991; Ochs et al., 2013; Pieper et al., 2013).

Mature B-cells could be classified into B-1 and B-2 subsets. The self-renewing B-1 cells secrete natural antibodies and contribute to T-cell independent responses. The mature B-2 cell population is constituted by follicular B-cells and the spleen-enriched marginal zone (MZ) subset (Ochs et al., 2013). The follicular B-cells express $CD21^{+}/CD23^{+}/IgM^{lo}/IgD^{hi}$ markers, respond to T-cell dependent antigens and could be differentiated into plasma and memory B-cells. Whereas, $CD21^{+}/CD23^{-}/IgM^{hi}/IgD^{lo}$ MZ B-cells are the main producers of IgM antibodies and preferentially respond to T-cell independent antigens (Appelgren et al., 2018). In response to antigen binding, upregulation of activation-induced cytidine deaminase (AID) is observed. AID mediates somatic hypermutation of the V region in both heavy and light chains, and immunoglobulin class switch recombination that replaces the μ constant region generating IgA, IgG or IgE antibodies. Ultimately, mature cells expressing different types of Ig are positively selected and produce long lived memory B-cells and plasma cells (Abbas et al., 1993; MacLennan, 1994; Ochs et al., 2013; Pieper et al., 2013).

1.2 Tyrosine kinases

TEC family kinases (TFKs) are non-receptor Protein Tyrosine Kinases (PTKs) that phosphorylate selected tyrosine residues on a substrate protein. Five members in the TFKs have been described, including Bruton Tyrosine Kinase (BTK), tyrosine kinase expressed in hepatocellular carcinoma (TEC), inducible T-cell kinase (ITK), resting lymphocyte kinase/T and X cell expressed Kinase (RLK/TXK) and bone marrow-expressed kinase (BMX) (Smith et al., 2001).

BTK and TEC play an important role in the B-cell development. BTK is a key component in the BCR signaling pathway and its expression has been reported in all hematopoietic cells with the exception of T- and plasma cells (Smith et al., 1994; Schmidt et al., 2004). TEC is expressed in platelets, erythrocytes, macrophages, neutrophils, endothelial, B- and T-cells, as reviewed by (Berglöf et al., 2015).

ITK is expressed in mast, NK- and T-cells (Schmidt et al., 2004). Mutations in the *ITK* gene in humans cause severe Epstein-Barr virus dysregulation (Ghosh et al., 2018) and ITK knock-out (KO) mice have decreased number of mature thymocytes and lowered ratio of $CD4^+/CD8^+$ cells (Liao and Littman, 1995). A more severe phenotype is observed in ITK/TXK mice, suggesting that TXK is also involved in T- and NKT-cell development (Broussard et al., 2006; Felices and Berg, 2008).

BMX is weakly expressed in macrophages and neutrophils but mainly in endothelial cells. It is involved in the response to ischemia, as well as survival and tumorigenicity of glioblastoma cancer stem cells (Cenni et al., 2012).

1.3 Bruton Tyrosine Kinase and B-cell development

The *BTK* gene is located on the X chromosome, region Xq22.1 (Vetrie et al., 1993). It encodes a 2.7 kb mRNA that is translated into a cytoplasmic PTK constituted by 659 amino acids (Tsukada et al., 1993; Vetrie et al., 1993; Smith et al., 1994). The structure of the BTK-protein consists of five different domains. From the C-terminal, BTK carries three *SRC homology* domains (SH), a SH1 or kinase domain, followed by SH2 and SH3 that interact with phosphorylated tyrosine residues and proline-rich stretches, respectively. *Pleckstrin homology* (PH) and *Tec homology* (TH) domains are located at the N-terminal and contain membrane-targeting function, a designated *Btk motif* and proline-rich stretch (Smith et al., 1994; Salim et al., 1996; Vihinen et al., 1997).

Mutations in the *BTK* gene in humans block the B-cell development and cause X-linked agammaglobulinemia (XLA). XLA is the most common form of inherited agammaglobulinemia in man and was reported for the first time in 1952 by Ogden C. Bruton (Bruton, 1952; Hendriks et al., 2011). Patients with either XLA or autosomal recessive agammaglobulinemia (ARA) present increased susceptibility to bacterial infections in the respiratory tract and also to enteroviral infections that could progress and cause death (Lederman and Winkelstein, 1985; Ochs et al., 2013). XLA and ARA are differentiated from other Ig deficiencies by the arrest in the B-cell development, during the pro-B to pre-B cell stage (Tsukada et al., 1993; Vetrie et al., 1993; Ochs and Smith, 1996; Noordzij et al., 2002). XLA patients are characterized by increased number of pro-B cells, decreased immature B-cells and almost total lack of mature B-cells, plasma cells and immunoglobulin production (Figure 1). Therefore, XLA patients are highly susceptible to extracellular bacterial and enteroviral infections (Ochs and Smith, 1996; Noordzij et al., 2002; Winkelstein et al., 2006).

On the other hand, a missense mutation in the mouse *Btk* gene causes X-linked immunodeficiency (Xid). This is a less severe defect, characterized by partial block in the pre-B cell stage of the B-cell development (Figure 1) (Scher, 1982; Khan et al., 1995). The Xid phenotype resembles the one found in the BTK KO mouse model (Khan et al., 1995). Even though Xid mice have less severe disease, similar phenotype to human XLA could be seen in mice with double KO for BTK and TEC (BTK/TEC dKO). This difference in phenotype may be explained by the ability of TEC to compensate BTK function in mice (Figure 1) (Ellmeier et al., 2000). However, results from TEC KO animals demonstrated that TEC is not essential for the B-cell development, since TEC-deficient mice did not show any characteristic phenotype (de Bruijn et al., 2017).

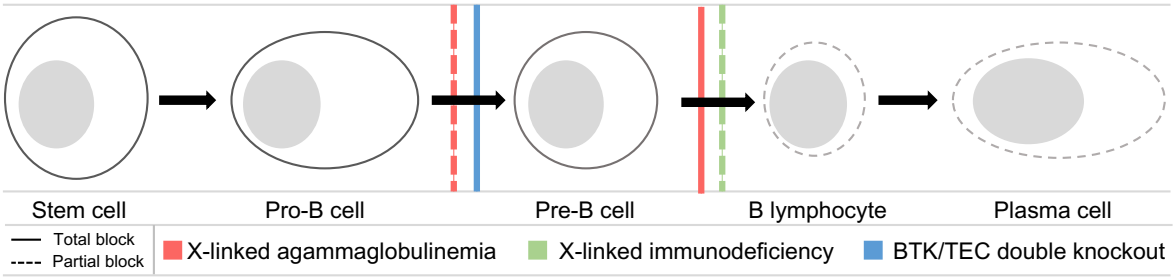

Figure 1. Scheme representing the B-cell differentiation block in XLA, Xid and BTK/TEC dKO mice.

Xid phenotype is characterized by small increase of $B220^{+}/CD43^{+}$ B-cells, lack of $CD5^{+}$ B1a cells in peritoneal cavity (Scher, 1982; Khan et al., 1995; Ellmeier et al., 2000) and reduction of two- to three fold of B-cell populations in spleen, with mainly IgM^{lo}/IgD^{hi} B-cells decreased by half (Khan et al., 1995; Ellmeier et al., 2000). In contrast to the reduction of all immunoglobulin classes observed in XLA patients, only reduced IgM, IgG2 and very low IgG3 levels are observed in Xid mice (Scher, 1982; Khan et al., 1995; Ellmeier et al., 2000).

The severe phenotypic alteration observed in the BTK/TEC dKO is caused by a stronger block in the B-cell development (Figure 1), evidenced by the BM accumulation of $CD43^{+}$ pro-B cells and the dramatically reduced frequency of peripheral $B220^{+}$ B-cells, particularly mature IgM^{lo}/IgD^{hi} B-cells in spleen (wild-type 49.8%; BTK/TEC dKO 7.7%) and lymph nodes (wild-type 63.1%; BTK/TEC dKO 3.9%) (Ellmeier et al., 2000). Similar to Xid mice, decreased serum IgM, IgG2a and IgG3 levels are observed in BTK/TEC dKO mice. In contrast to the minor structural alterations observed in spleen from Xid mice, BTK/TEC dKO has fewer clearly defined small follicular structures, unclear or discontinued $MOMA\text{-}1^{+}$ MZ macrophage layer and reduced frequency of germinal centers (Khan et al., 1995; Ellmeier et al., 2000).

1.4 BTK in the B-cell receptor signaling pathway

Each B-cell expresses a unique BCR, which is composed of two identical immunoglobulin heavy and light chains coupled by a disulfide-linked heterodimer, Igα/Igβ (CD79a/CD79b) co-receptor (Hombach et al., 1990; Radaev et al., 2010). In the cytoplasmic domain of each CD79 molecule, there is an immunoreceptor tyrosine-based activation motif (ITAM), which upon BCR stimulation gets phosphorylated by a SRC-family kinase, predominantly LYN (Rolli et al., 2002; Tolar et al., 2008). This leads to the activation of phosphatidylinositol-3-kinase (PI3K) and spleen tyrosine kinase (SYK). PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP_3) that allows translocation of BTK onto the cell plasma membrane (Salim et al., 1996). Activated LYN phosphorylates BTK on the Y551 residue, leading to activation of BTK. This is followed by autophosphorylation of the Y223 residue in the SH3 domain (Figure 2a).

Phospholipase C-γ2 (PLCγ2) is activated by BTK and a signalosome composed by SYK, BTK, PLCγ2 and the protein adaptor BLNK/SLP-65 is then produced. Phosphatidylinositol-3,4,5-triphosphate is cleaved by PLCγ2 into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) (Herrera and Jacobsen, 2014). This increases the levels of intracellular calcium resulting in the activation of protein kinase Cβ (PKCβ) and transcription factors involved in the regulation of B-cell proliferation, differentiation, survival and migration (Figure 2a) (Herrera and Jacobsen, 2014).

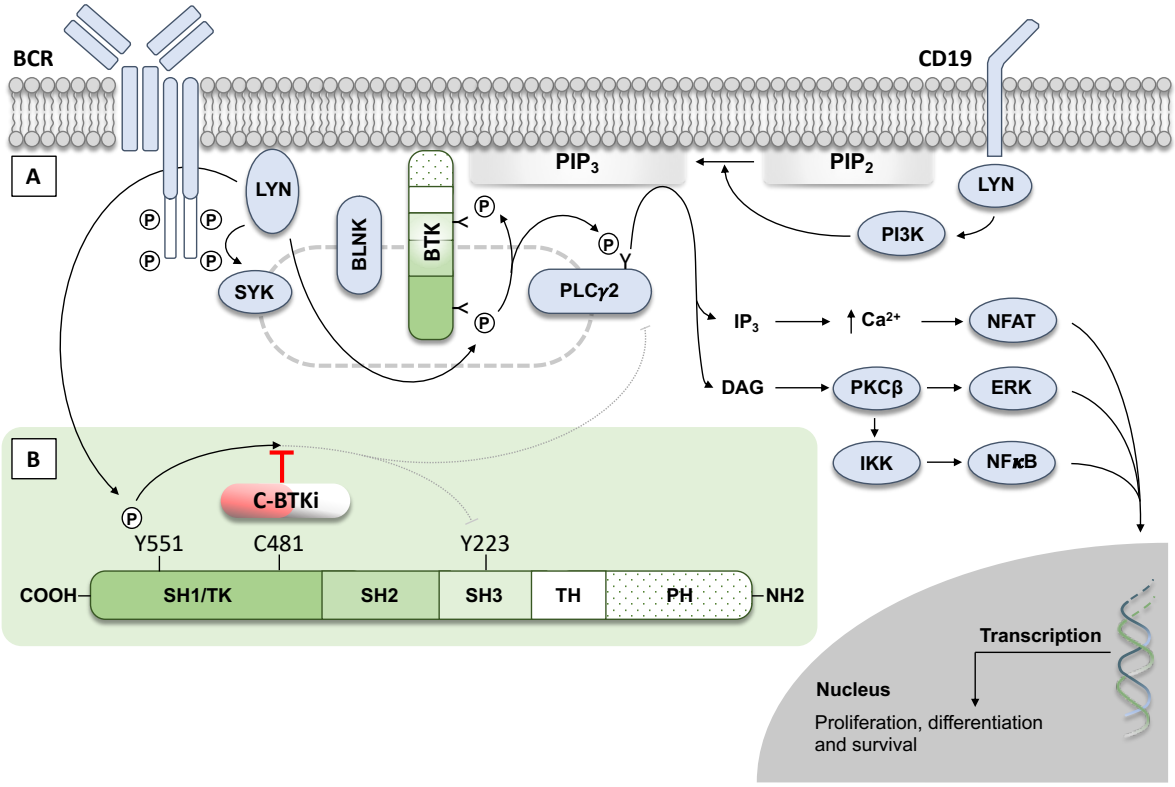

Figure 2. Schematic representation of BTK and BTK-inhibitors role in the BCR signaling pathway. (A) BCR-BTK signaling pathway. (B) Inhibition of the BTK´s catalytic activity caused by covalent BTK inhibitors. BTK protein is depicted with all five domains (SH1-3, TH and PH), two phosphorylation sites Y551/Y223 and the corresponding cysteine-481 inhibitor binding site. C-BTKi indicates covalent BTK inhibitor; C, cysteine; Y, tyrosine; P, phosphorylation.

1.5 BTK outside the B-cell receptor signaling pathway

The role of BTK in other cell compartments, aside from B-lymphocytes, has emerged from the study of XLA patients and the BTK-deficient mouse model (Schmidt et al., 2004). In macrophages, BTK gets activated upon lipopolysaccharide (LPS) stimulation leading to the tumor necrosis factor alfa (TNFα) production (Horwood et al., 2003). Monocytes and macrophages lacking BTK show impaired chemotaxis/phagocytic function (Braga Amoras et al., 2003) and reduced secretion of the proinflammatory cytokines TNFα and interleukin-1ß (IL-1ß), respectively (Mukhopadhyay et al., 2002).

Mast cells have a key role in the initiation of allergic reactions, they are primarily activated by cross-linking of the high-affinity IgE receptor, which leads to cell degranulation, histamine and cytokine secretion (Schmidt et al., 2004). BTK-deficient mast cells have impaired secretion of proinflammatory cytokines such as IL-2 and TNFα (Hata et al., 1998), show spontaneous degranulation in the absence of stimulation and increased proliferation potential (Dvorak et al., 1996; Kawakami et al., 2000).

BTK-deficient megakaryocytes, in mice, display a reduced intracellular Ca^{2+} mobilization (Pasquet et al., 2000) and platelets from XLA carriers have reduced aggregation response upon collagen induction (Quek et al., 1998; Berglöf et al., 2015). However, XLA patients do not suffer from bleeding and present normal megakaryocyte development, suggesting an alternative compensatory mechanism (Berglöf et al., 2015). In addition, an important role of BTK has also been described in the survival of neutrophils and activation of NK-cells and dendritic cells (Bao et al., 2012; Honda et al., 2012; Lougaris et al., 2014).

1.6 BTK inhibition in B-cell malignancy

The central role of BTK in tumorigenesis is still debated and not well understood. Gain-of-function substitutions in BTK were not reported in patients with B-cell malignancies (Smith, 2017b). However, it was described that overexpression and constitutive BTK phosphorylation lead to activation of PLCγ2 and downstream molecules, which promote upregulation of pro-survival signals and migration in chronic lymphocytic leukemia (CLL) cells (Herman et al., 2011). In mantle cell lymphoma (MCL) cells BTK was shown to be important for adhesion and chemotaxis (Chang et al., 2013).

In animal models, it was demonstrated that blocking of the BCR signaling pathway may cause lymphomas, by combining and inactivating mutations in both BTK and BLNK (Kersseboom et al., 2003). However, this scenario is very unexpected in patients due to the requirement of multiple mutations in the DNA (Smith, 2017b). While patients with XLA only show clinically relevant defects in B-cells, other BTK-containing cells in the microenvironment may still promote tumor growth and hence inhibition of BTK could play a role apart from an effect on the tumor B-cell *per se*. For instance, BTK inhibition by ibrutinib was demonstrated in myeloid

derived suppressor cells, which are known as critical contributors of tumor evasion and promoters of immune suppression (Stiff et al., 2016).

On the other hand, it is known that BTK has an essential role in the proliferation, migration and survival of malignant B-cells (De Rooij et al., 2012, 2015). Therefore, BTK inhibitors (BTKis) were developed and have revolutionized the treatment of B-cell malignancies (Pal Singh et al., 2018; Lucas and Woyach, 2019).

1.6.1 Irreversible BTK inhibitors

Ibrutinib (PCI-32765, Imbruvica®) is the most studied BTKi (Honigberg et al., 2010; Herman et al., 2011; Byrd et al., 2013; Burger et al., 2015; Smith, 2017b) and first-in-class irreversible binder that has been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Ibrutinib is indicated for the treatment of adults with at least one prior therapy in MCL, previously treated or untreated CLL/small lymphocytic leukemia (SLL) and Waldenström macroglobulinemia (WM). FDA also indicates ibrutinib for previously treated marginal zone lymphoma (MZL) patients and chronic graft-versus-host disease (cGVHD) (FDA., 2020).

Ibrutinib blocks the catalytic activity of BTK by forming a covalent bond between its acrylamide moiety and the thiol group of the cysteine (C) 481 located in the ATP-binding site of the BTK (Figure 2b and 3) (Johnson et al., 2016; Chen et al., 2018; Gehringer and Laufer, 2019). Two additional hydrogen bonds were described with the Glu475 and Met477 residues in the BTK kinase domain (Figure 3) (Johnson et al., 2016). Ibrutinib is a small-molecule BTK inhibitor that reaches maximum serum concentration of ~0.5 μM, one to two hours after oral administration (420 mg, once-daily dose). It is rapidly absorbed and has a half-life of 4 to 6h (Advani et al., 2013; Marostica et al., 2015). Inhibition of BTK by ibrutinib showed, overall response rate (ORR) of 83 to 97% in relapsed/refractory (R/R) CLL patients, (O'Brien et al., 2016; Ahn et al., 2018) 90 to 92% in rituximab-refractory WM patients (Dimopoulos et al., 2017; Treon et al., 2018; Castillo and Treon, 2020) and 68 to 70% in R/R MCL patients (Wang et al., 2013; Rule et al., 2017).

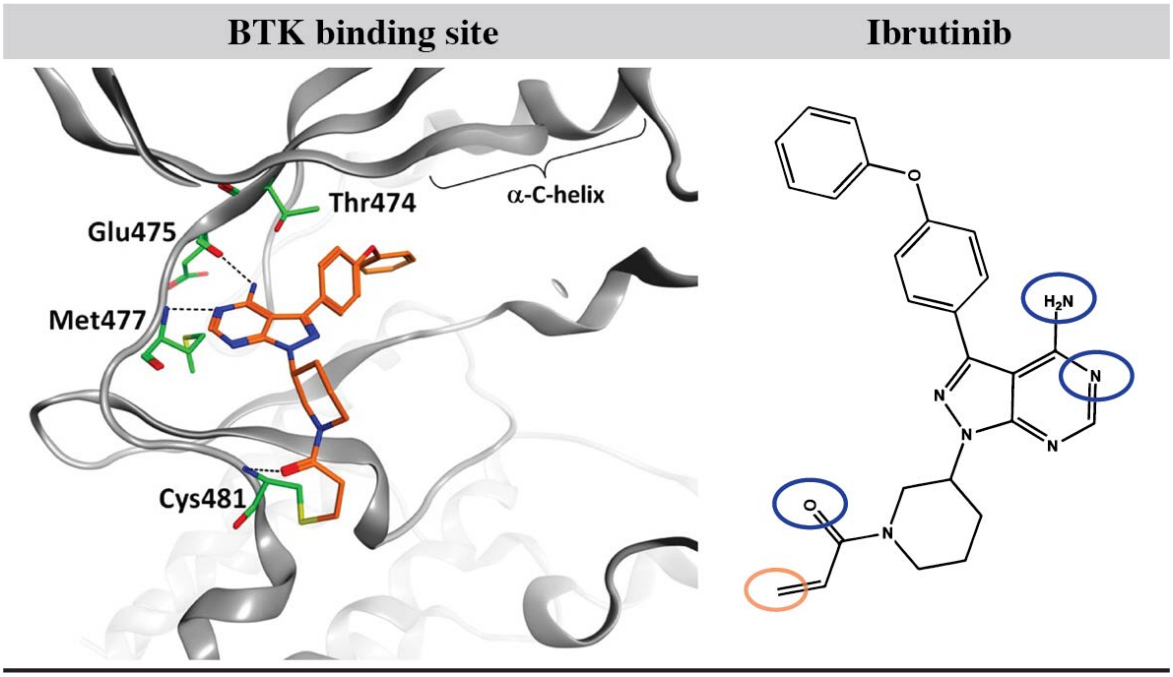

Figure 3. Molecular model of ibrutinib covalently bound to BTK. Right panel shows ibrutinib chemical structure, blue and orange ovals indicate sites of formation of hydrogen and covalent bond, respectively. Adapted with permission from Johnson, A. *et al*. (2016). Battling Btk Mutants with Noncovalent Inhibitors That Overcome Cys481 and Thr474 Mutations. ACS Chem. Biol. 11, 2897–2907. Copyright 2016 American Chemical Society.

Due to the therapeutic success of ibrutinib but also due to the fact that this drug causes side effects, a second generation of more selective BTKis was developed. Acalabrutinib (ACP-196, Calquence®), zanubrutinib (BGB-3111, Brukinsa®) and tirabrutinib (Velexbru®) are examples and new FDA-approved BTKis.

Acalabrutinib also covalently binds to the C481 residue in the BTK kinase domain, and has been reported to show higher selectivity and reduced number of off-targets compared with ibrutinib (Table 1) (Byrd et al., 2016). Acalabrutinib is FDA- and EMA-approved for the treatment CLL/SLL patients and only FDA approved for MCL (Markham and Dhillon, 2018; FDA., 2019b). Its efficacy has been evaluated in CLL and other B-cell malignancies and a high ORR has been shown in patients with R/R disease (Markham and Dhillon, 2018; Wang et al., 2018; Byrd et al., 2020b). Zanubrutinib is also a next generation BTK inhibitor suggested to be more selective than ibrutinib (Table 1) (Guo et al., 2019). It has shown potent clinical activity and high ORR with minimal off-target effects in phase 1/2 studies, when used for the treatment of MCL and CLL/SLL patients (Seymour et al., 2017; Tam et al., 2018). Zanubrutinib was FDA approved for MCL, EMA approved in pretreated WM patients, and in China the National Medical Products Administration (NMPA) approved zanubrutinib for CLL/SLL and pretreated MCL patients (FDA., 2019a). Tirabrutinib got approval by the Pharmaceuticals and Medical Devices Agency (PMDA) in Japan for treatment of R/R primary central nervous system lymphoma and WM (Dhillon, 2020). This is a highly expanding field and only for the last three mentioned BTKis more than 100 clinical trials have been registered at the ClinicalTrials.gov (Table 1).

Table 1. Selectivity and adverse events in approved BTKis. Only kinases having a cysteine in the ATP-binding site corresponding to the C481 in BTK are displayed in the table; A, active; C, completed; AF, atrial fibrillation.

BTK inhibitors	Number of active (total) clinical trials	Inhibited kinases	Reported adverse events Any grade (≥3)
PCI-32765 Ibrutinib Imbruvica®	236 (364) Phase III A(31) C(13) Phase IV A(3) C(0)	BTK, BLK, BMX/ETK, EGFR, ERBB2/HER2, ERBB4/HER4, ITK, JAK3, RLK/TXK, TEC (Byrd et al., 2016; Guo et al., 2019; Liclican et al., 2020)	AF: 2 – 17% (2 – 9%) Rash: 2 – 47% (2 – 3%) Diarrhoea: 3 – 65% (2 – 16%) (Farooqui et al., 2015; Munir et al., 2019; Burger et al., 2020)
ACP-196 Acalabrutinib Calquence®	82 (103) Phase III A(13) C(1)	BTK, BMX/ETK, ERBB4/HER4 (Byrd et al., 2016; Liclican et al., 2020)	AF: 4 – 7% (≤3%) Rash: 6 – 25% (0.6 – 2%) Diarrhoea: 17 – 58% (0.6 – 5%) (Awan et al., 2019; Byrd et al., 2020a)
BGB3111 Zanubrutinib Brukinsa®	40 (52) Phase III A(8) C(0)	BTK, BLK, BMX/ETK, EGFR, ERBB4/HER4, ITK, RLK/TXK (Guo et al., 2019)	AF: ≤5% (<2%) Rash: 11 – 33% (NR) Diarrhoea: 8.3 – 30% (≤3%) (Tam et al., 2020; Trotman et al., 2020)
ONO-4059 Tirabrutinib Velexbru®	3 (9)	BTK, BMX/ETK, TEC (Liclican et al., 2020)	AF: 0 / NR (0 / NR) Rash: 19 – 44% (0 – 6%) Diarrhoea: 7.4 – 44% (0 – 7%) (Rule et al., 2020; Sekiguchi et al., 2020)

1.6.2 Adverse events and resistance to irreversible BTK inhibitors

Ibrutinib was described as a safe BTK inhibitor, well tolerated and with rapid and durable response (Byrd et al., 2013, 2015; Winqvist et al., 2019). Ibrutinib has high selectivity for BTK, however, off-target inhibition has been reported in other kinases, which have a cysteine residue located in the ATP-binding site (Table 2), reviewed by (Berglöf et al., 2015). Despite the good results of ibrutinib in the treatment of the B-cell malignancies, adverse events and the emergence of resistance remain critical concerns. The most commonly observed adverse events that lead to ibrutinib discontinuation are atrial fibrillation (AF), bleeding, infections diarrhoea and rash (Table 1) (Byrd et al., 2015; Jain et al., 2015, 2017; Mato et al., 2018; Munir et al., 2019).

Table 2. Alignment of kinases that have a cysteine residue in the ATP-binding site, kinase cell expression and its possible association with the occurrence of the adverse event. Underlined, kinases that belong to the TEC family kinases; AF, atrial fibrillation. Inspired and adapted from Berglöf, A., *et al*. (2015). Targets for Ibrutinib Beyond B Cell Malignancies. Scand. J. Immunol. 82, 208–217. doi:10.1111/sji.12333.

Protein	Cysteine in the ATP-binding site, corresponding to the C481 in BTK	Cell expression and possible association with the occurrence of BTKi adverse events
BTK	T E Y M A N G **C** L L N	**Infection**: B-cells, macrophages and neutrophils **Bleeding**: platelets
ITK	F E F M E H G **C** L S D	**Infection**: T-cells
TEC	T E F M E R G **C** L L N	**Infection**: B-, T-cells, macrophages and neutrophils **Bleeding**: platelets **AF**: cardiomyocytes
BMX	T E Y I S N G **C** L L N	**Unclear**
RLK/TXK	T E F M E N G **C** L L N	**Infection**: T-cells
BLK	T E Y M A R G **C** L L D	**Unclear**
JAK3	M E Y L P S G **C** L R D	**Unclear**
EGFR	T Q L M P F G **C** L L D	**Rash and diarrhoea**: epithelial cells
ERBB2/HER2	T Q L M P Y G **C** L L D	**AF**: cardiomyocytes
ERBB4/HER4	T Q L M P H G **C** L L E	**AF**: cardiomyocytes

Higher risk of developing cardiovascular disease has been associated with ibrutinib treatment; the 5 years follow-up RESONATE-2 study reported AF of any grade in 16% of the treated patient, of which 5% developed AF ≥grade 3 (Table 1) (Burger et al., 2020). Results from a real-world analysis including 616 CLL-patients confirmed this association and reported AF in 12% front-line and 25% R/R treated patients (Mato et al., 2018). Development of hypertension or worsened high blood pressure has also been observed in more than 3/4 of ibrutinib treated patients (Dickerson et al., 2019). These cardiovascular events have been attributed to possible blocking of cardiac PI3K-Akt signaling pathway through off-target inhibition of TEC, ERBB2/HER2 or ERBB4/HER4 (Table 2) (Albini et al., 2011; Milano et al., 2014; Berglöf et al., 2015). Interestingly, results from the head-to-head ASPEN-study comparing the more selective BTKi zanubrutinib versus ibrutinib in WM patients, reported ≥10% higher incidence of AF and increased hypertension in ibrutinib treated patients, compared with zanubrutinib (Tam et al., 2020). Other phase III studies, comparing zanubrutinib versus ibrutinib in R/R

CLL/SLL (ALPINE) (Hillmen et al., 2020) and acalabrutinib versus ibrutinib monotherapy (NCT02477696), are ongoing and will provide further comparison.

Increased risk for bleedings is another ibrutinib associated adverse event (Lipsky et al., 2015; Wang et al., 2015). An integrated analysis of 15 ibrutinib clinical studies, reported an overall incidence of 40% bleeding of any grade, 4% of the patients developed major haemorrhage and in 1% of the patients this led to treatment discontinuation (Brown et al., 2019). Low aggregation response and reduced adhesion have been observed in platelets from ibrutinib treated patients that experienced bleedings (Levade et al., 2014; Kamel et al., 2015), which correlates with the absence of response and highly reduced aggregation upon collagen stimulation in platelets from BTK/TEC dKO mice (Atkinson et al., 2003; Levade et al., 2014; Berglöf et al., 2015; Kamel et al., 2015). These findings suggest that inhibition of both BTK and TEC is involved in the increased risk of bleeding (Table 2). However, inhibition of these kinases cannot be pointed out as the only cause of this adverse event, since bleedings are also found upon treatment with BTKis that are less prone to inhibit TEC, such as acalabrutinib and zanubrutinib.

The risk of infections also increases in ibrutinib treated patients and the risk is specially high during the first 6 months of treatment (Varughese et al., 2018). The results of a systematic review covering 48 clinical trials, showed that infections occur in 56% of patients receiving single-ibrutinib treatment and that >25% of the treated patients progressed to grade 3-4 (Tillman et al., 2018). Invasive fungal infections, specially caused by Aspergillus species, are the most frequently identified opportunistic infections (Ghez et al., 2018; Tillman et al., 2018; Rogers et al., 2019). The mechanism underlying this adverse event could be associated with both on- and off-target kinase inhibition (Table 2). Neutropenia and reduction in the macrophage response are both common side effects found in treated patients (Rogers et al., 2017; Ghez et al., 2018). Macrophages express three TEC family members BTK, TEC and BMX, and BTK-deficient macrophages have impaired phagocytic function, chemotaxis and reduced secretion of TNFα (Weil et al., 1997; Schmidt et al., 2004).

Rash and diarrhoea are also common adverse events occurring in patients treated with irreversible BTKis (Table 1). The analysis of dermatological toxicity carried out (Sibaud et al., 2020) reported a higher frequency of rash in ibrutinib treated patients (13-27%), compared with acalabrutinib 15-18% and zanubrutinib 13-18%. Similar to the observed for rash, the frequency of diarrhoea is higher in ibrutinib 3-65% (≤16% ≥grade 3) than acalabrutinib 17-58% (≤5% ≥grade 3) (Table 1), and reduced number of cases were reported for zanubrutinib (21% versus 32%) in the head-to-head ASPEN-study (Tam et al., 2020).

Furthermore, some of the ibrutinib treated patients develop resistance. The most common cause of ibrutinib resistance is the cysteine-481 to serine substitution (C481S), which prevents the covalent binding and reduces the efficacy of the irreversible BTKis (Furman et al., 2014; Woyach et al., 2017; Estupiñán et al., 2020). In a cohort of 61 ibrutinib treated CLL patients, clonal shift was reported in 31% of the patients, after 1 year of treatment initiation (Landau et al., 2017), and ~60% (published data: 57 – 64.4%) of ibrutinib treated patients with disease progression present resistance variations, mainly in BTK (Woyach et al., 2017; Quinquenel et

al., 2019). Other less frequent variants, different than C481S, were reported in CLL patients including C481A, C481F, C481G and C481R (Furman et al., 2014; Landau et al., 2017; Woyach et al., 2017; Quinquenel et al., 2019).

Substitutions in PLCγ2 and the gatekeeper residue of BTK, are also involved in the development of resistance to BTKis. Gain-of-function substitutions in PLCγ2, like S707Y and R665W, generate a constitutively active form, which does not need to be activated by BTK (Zhou et al., 2012; Woyach et al., 2014). T474I and T474S substitutions in the BTK gatekeeper residue have also been reported in CLL ibrutinib resistant patients, which already carry a previous C481S mutation. The gatekeeper residue in BTK is located in the regulatory spine of the kinase domain and maintains a rigid and linear structure, crucial for the catalytic activity of BTK (Chopra et al., 2016).

The involvement of the gatekeeper residue in the resistance to both irreversible and the more recently developed reversible (non-covalent) BTKis was described (Wang et al., 2019). Computational structure modeling demonstrated that T474M substitution affects the binding of both irreversible and reversible BTKis. The reversible inhibitor RN486 was used in the analysis and it was shown that it binds to wild-type BTK using a hydrogen-bond network between K430, T474 and G414. It was also demonstrated that introducing methionine in the gatekeeper residue 474 of the BTK disrupted the hydrogen-bond network (Wang et al., 2019).

Inhibitors of other kinases different than BTK are also affected by substitutions in the gatekeeper residue. In BCR-ABL positive chronic myeloid leukemia (CML) patients, T315I substitution causes resistance to imatinib (Shah et al., 2002). Crizotinib, an anaplastic lymphoma kinase (ALK)-inhibitor is affected by the gatekeeper variant L1196M (Kay and Dehghanian, 2017). Resistance to the epidermal growth factor receptor (EGFR)-inhibitors, gefitinib, erlotinib and afatinib, is caused by the T790M substitution in lung cancer cells (Jia et al., 2016).

1.6.3 Reversible BTK inhibitors

The reversible BTKis also known as non-covalent BTKis, are a new generation of selective inhibitors that are not affected by the cysteine to serine substitution. Molecular modeling analysis of the reversible BTKis RN486, fenebrutinib and CGI-1761 shows a unique binding mode in the H3 pocket of the BTK kinase domain, which is independent of the C481 and confers a highly selective inhibition of BTK that overcomes the resistance caused by the C481S substitution (Figure 4) (Estupiñán et al., 2020, 2021).

Fenebrutinib (GDC-0853) is a highly selective BTK inhibitor, previously evaluated in healthy donors, resistant B-cell lymphoma and CLL patients (Byrd et al., 2018; Herman et al., 2018). Equivalent blocking of Y223 phosphorylation was observed when fenebrutinib was used in WT- and C481S-BTK transfected HEK 293T cells. Reduction of CCL3 production and cell death were also observed in C481S patient-clones treated with fenebrutinib (Reiff et al., 2018).

Fenebrutinib is currently under evaluation in phase 1 and 2 clinical trials (Chan et al., 2020), and more than 10 clinical trials have been reported in the NIH clinical trials database.

Other reversible BTKis have been developed and showed potential in pre-clinical studies (Lou et al., 2015; Cohen et al., 2020; Estupiñán et al., 2021). For example, CGI-1746 was evaluated in rheumatoid arthritis and multiple myeloma mouse models and it was shown that it reduces cell proliferation and tumor growth (Di Paolo et al., 2011; Gu et al., 2017). RN486 was shown to prevent type I and type III hypersensitivity responses and inhibit both joint and systemic inflammation in rheumatoid arthritis mouse models (Xu et al., 2012; Lou et al., 2015).

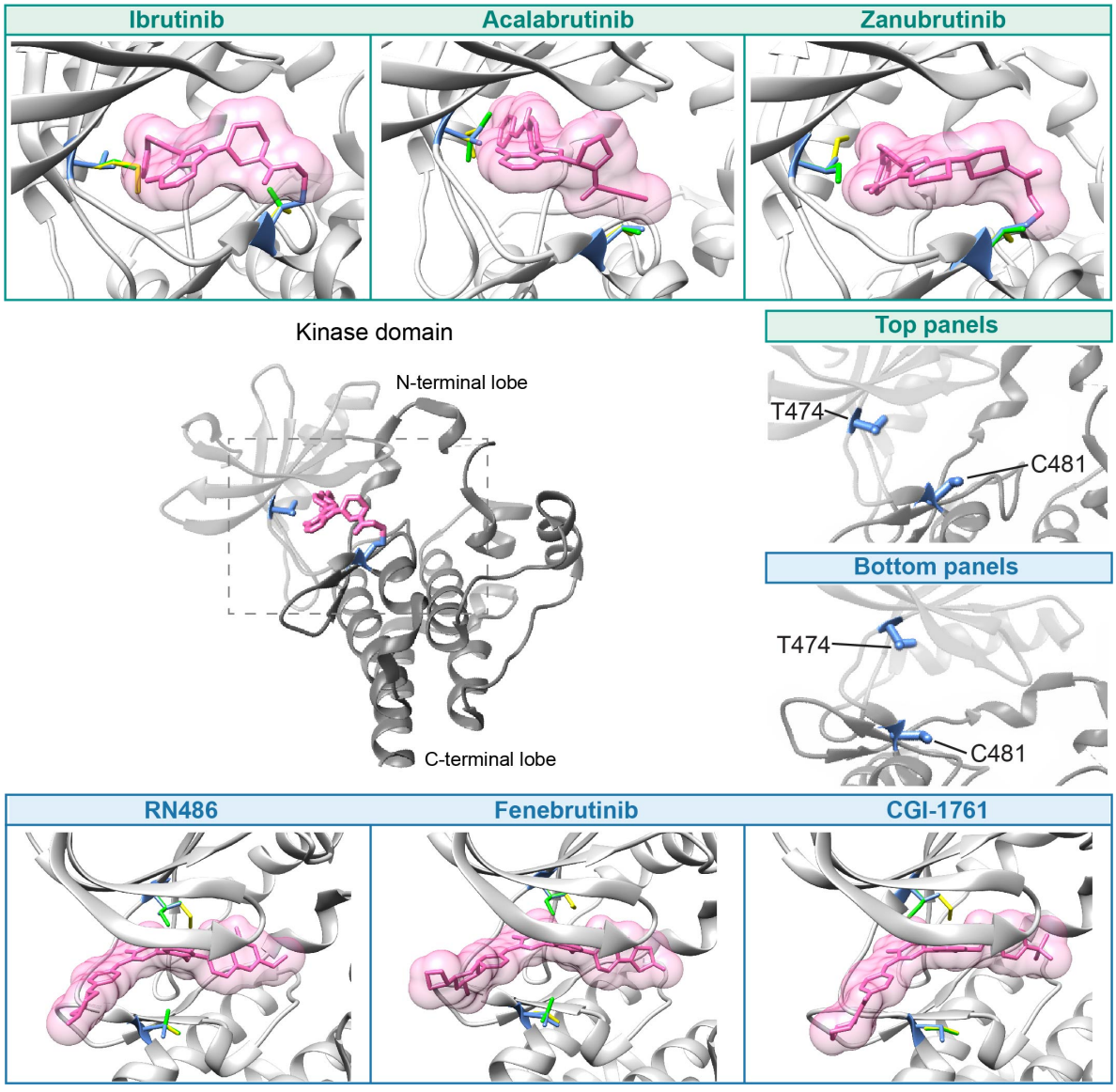

Figure 4. Computational docking model of BTK with reversible and irreversible BTK inhibitors. Left-center, kinase domain with ibrutinib (pink) in the catalytic site; N-terminal lobe (light gray); C-terminal lobe (dark gray). Top row shows binding of irreversible inhibitors and the bottom row of reversible inhibitors. Original residues T474 and C481 are in blue; substitutions at 474 methionine (yellow) and isoleucine (green), at 481 serine (yellow) and threonine (green). Adapted from Estupiñán, H. Y., *et al*. (2021). BTK gatekeeper residue variation combined with cysteine 481 substitution causes super-resistance to irreversible inhibitors acalabrutinib, ibrutinib and zanubrutinib. Leukemia, Online ahead of print. doi:10.1038/s41375-021-01123-6

2 RESEARCH AIMS

The overall aim of this thesis was to perform *in vitro* and *in vivo* studies of mutated BTK and the response to both reversible and irreversible BTKis.

Specific aims:

2.1 Paper I

Phenotypic analysis of the mouse model with C481 to serine substitution in the BTK and assessment of the effects of BTKis on B- and T-lymphocytes.

2.2 Paper II

Evaluation of *in vitro* susceptibility of gatekeeper and combined gatekeeper/C481 BTK variants to both reversible and irreversible BTKis.

3 MATERIALS AND METHODS

This chapter briefly describes some of the relevant methodologies chosen for the projects discussed in this thesis. Full description of the materials and methods can be found in the respective publications.

3.1 Ethics

Studies were approved by the local ethics committee and performed in accordance with the ethical permissions ID1679 and Dnr: S49-13.

3.2 Cell sources

In **paper I**, all the experiments were performed using primary cells obtained from the C481S mouse model. In **paper II**, expression and catalytic activity of BTK were evaluated in lymphoid and non-lymphoid cell lines.

The BTK knock-out lymphoid B7.10 cell line was chosen as a relevant biological model for our study. It was generated from the DT40 chicken B lymphoma cell line and generously provided by Dr. T. Kurosaki (Nawaz et al., 2008). The non-lymphoid cell lines COS-7 (African green monkey fibroblast-like kidney cells) and HEK-293T (human embryonic kidney cells) were purchased from the American Type Culture Collection. These cell lines were selected because they allowed analysis without the influence of endogenous BTK.

3.3 Plasmids and transient transfections

Plasmids were generated with single and double BTK variants and transient transfections were performed only in **paper II**.

In the non-lymphoid cell lines COS-7 and HEK-293T plasmids were transfected by using Polyethylenimine (PEI) (Polyscience, Inc., Warrington, PA, USA). Whereas, in the B7.10 cell line we carried out electroporation by using the Neon transfection system (Life Technologies, Carlsbad, CA, USA). A single pulse was performed with 2000 V for 20 milliseconds.

3.4 Western blot

In **paper I** and **II**, we investigated BTK protein expression, catalytic activity and its inhibition by western blot. First, we measured the expression of total proteins and compared it with wild-type. Then, we analyzed the corresponding phosphorylated sites in BTK and PLCγ2.

Phosphorylation of Y551-BTK residue indicates BTK-activation, whereas phosphorylation of Y223-BTK and Y753-PLCγ2 reflects catalytic activity of BTK.

Primary cells or transfected cell lines were collected after serum-free medium starvation and then stimulated for 5 min at room temperature. Two stimulation procedures were carried out.

Primary B-cells and B7.10 cells were stimulated by using 4 mM H_2O_2 with either 10 μg/mL anti-mouse IgM (1022-01) or mouse anti-chicken IgM (M-4) respectively. COS-7 and HEK-293T cells were both stimulated with FBS (2%) and pervanadate [0.02% H_2O_2, 1.6% Tyrode's salt solution (Sigma-Aldrich) and 0.22 mM Na_3VO_4 (Sigma-Aldrich)]. When required, BTK inhibition and washout experiments were performed prior to activation.

Whole-cell lysates were obtained by using modified RIPA buffer containing phosphatase inhibitor and heated at 65°C for 5 minutes with sample buffer. Samples were run at 120V for 2h in Bis-Tris (4-12%) protein gels and MES SDS running buffer. Proteins were transferred to the nitrocellulose membrane by using iBlot system and membranes were blocked and incubated with primary and secondary antibodies as previously described (Hamasy et al., 2017). Membranes were scanned with the Odyssey infrared imaging system according to the manufacturer´s protocol.

3.5 BTK inhibitors

In both **paper I** and **II**, all BTKis used for *in vitro* inhibition of the BTK catalytic activity were dissolved in dimethyl sulfoxide (DMSO) and prior to each experiment a fresh dilution was prepared in serum-free medium or phosphate buffered saline (PBS).

In **paper I**, for *in vivo* treatment, ibrutinib solution in drinking water containing 3% (2-Hydroxypropyl)-β-cyclodextrin (H107; Sigma-Aldrich) was prepared reaching a final concentration of ca 0.3 mg/ml.

3.6 Animal studies

In **paper I**, we published the generation of a mouse model with a cysteine 481 to serine substitution in the exon 15 of the *BTK* gene, by using CRISPR/Cas9-mediated gene editing. The mouse model was obtained from Taconic Biosciences and the targeting strategy was based on National Center for Biotechnology Information (NCBI) transcript NM_013482.2. Most of the experiments were performed in 9-12 weeks old mice, including age- and sex- matched wild-type controls. The phenotype of aged mice (16 or 20 months old) was also analyzed.

3.6.1 Flow cytometry

Flow cytometry is a technique that allows multi-parametric analysis of fluorescence-labeled single-cell suspensions. Cell populations can be defined base on their fluorescent characteristics and sorted for further analysis such us cell-viability or protein expression. Fluorescent reagents most commonly used in flow cytometry are fluorochrome-conjugated antibodies, fluorescent expression proteins, DNA binding and viability dyes.

In **paper I**, for the phenotypic analysis of the C481S mouse model we performed a battery of flow cytometry-based experiments that allowed us to determinate cellularity, cell distribution and expression of activation markers in selected hematopoietic cell compartments. We focused our analysis on the B-cell compartment, mainly in the B-cell development and distribution of peripheral B-cell subsets. However, hematopoietic progenitors and other non-B-lineage leukocytes were also analyzed.

Cells were obtained from dissected and crushed organs, Fc-blocked by using anti-CD16/31 and stained with fluorochrome-conjugated antibodies as previously described (Bouderlique et al., 2019). In order to efficiently separate positive from negatives populations, fluorescence minus one (FMO) controls were included in all experiments. We used propidium iodide to discriminate dead cells and data were acquired and analyzed on FACSAria IIu (BD Biosciences) and Flowjo 9.9.6 respectively.

3.6.2 Cell stimulation, inhibition and cell-viability assay

In **paper I**, cell-viability and CD25/CD69 expression were evaluated upon B- and T-cell stimulation with/without BTK inhibitors.

For B- and T-lymphocyte activation, 2×10^6 splenocytes were seeded in a 24-well plate, containing either IMDM medium with 15% FCS and 50 mM β-mercaptoethanol for B-lymphocytes or RPMI 1640 medium with 10% FCS for T-lymphocytes. B-lymphocytes were stimulated by adding 20 μg/mL of anti-mouse IgM (1022-01) and T-lymphocytes by coating the 24-well plate with 10 μg/mL of anti-CD3e (145- 2C11).

BTK inhibition was performed five minutes after stimulation and cells were cultured for 30 hours at 37°C. Then, cells were harvested and stained with fluorochrome-conjugated antibodies. CD25/CD69 expression and cell-viability were evaluated by using flow-cytometry based protocols. LIVE/DEAD Aqua (Invitrogen) was used for dead cell discrimination, data were acquired and analyzed on FACSAria IIu (BD Biosciences) and Flowjo 9.9.6 respectively.

3.6.3 Enzyme-linked immunosorbent assay (ELISA)

Reduced IgM and almost total lack of IgG3 antibody levels are found in BTK-deficient mice (Scher, 1982; Khan et al., 1995; Ellmeier et al., 2000). In **paper I**, to investigate if this anomaly

was present in our C481S mouse model, we performed ELISA for total IgM, IgG and IgG3 antibodies. Also, we have studied the production of anti-DNA antibodies as an indication of autoimmune response, which has been frequently seen in aged mice with dysregulated immunoglobulin production (Scher, 1982; Bygrave et al., 2004; Nusser et al., 2014).

For total immunoglobulin quantification, serum dilutions were prepared and incubated on anti-IgG (H+L) coated plates. Then, horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG, IgM, and IgG3 antibody was added to the plate and incubated for 1 hour at 37°C. Developing, blocking and reading were performed by using 3,3',5,5′-tetramethylbenzidine (TMB) substrate set, sulfuric acid (1 M) and Bio-Rad microplate reader (450 nm) respectively. For anti-DNA reaction, serum was incubated on plates coated with methylated bovine serum albumin and calf thymus DNA. Then, anti-mouse immunoglobulin-HRP was added on the plates and treated as described previously.

3.6.4 Immunohistochemistry

In **paper I**, to evaluate that our C481S mouse model did not have altered structure of B-cell compartments in peripheral lymphoid organs, we compared C481S with wild-type mice by performing immunohistochemistry in spleen and lymph nodes.

Spleens and lymph nodes were frozen and sectioned by using cryostat microtome. Then, 8-mm-thick slides were prepared, dried and fixed in acetone. Slides were serum-blocked and stained with biotin- or fluorophore-conjugated antibodies. Afterward, sections were washed and incubated with fluorophore-conjugated streptavidin. Slides were prepared with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific), data were acquired with a confocal microscope (Zeiss LSM880) and recorded with LSM Image software.

4 RESULTS AND DISCUSSIONS

4.1 Paper I

Ibrutinib BTK-inhibition has revolutionized the treatment strategy for B-cell malignancies (Honigberg et al., 2010; Advani et al., 2013; Lucas and Woyach, 2019) and demonstrated a remarkable therapeutic effect even during long-term follow-up (Byrd et al., 2019). However, the emergence of resistance (Woyach et al., 2017; Quinquenel et al., 2019) and the ibrutinib off-target binding adverse events are of concern (Jain et al., 2017; Mato et al., 2018; Munir et al., 2019). In this study, we generated a mouse model carrying the C481 to serine substitution (C481S), which is the most common ibrutinib resistant variant abrogating the covalent binding of ibrutinib to the thiol group located at the ATP-binding site (Furman et al., 2014; Woyach et al., 2017).

Lack of functional BTK in mice causes partial arrest of the B-cell development leading to an increase of proB-cells and decrease of immature and mature B-cells (Khan et al., 1995; Ellmeier et al., 2000). Peripheral B-cell populations are affected in these mice. Reduction of both IgM^{+} and mature IgM^{lo}/IgD^{hi} B-cells is observed in spleen and lack of $CD5^{+}$ B1a cells in peritoneal cavity (Scher, 1982; Khan et al., 1995; Ellmeier et al., 2000). To investigate if the C481S substitution has impact on the B-cell development, we used flow cytometry-based protocols for the identification of B-cell progenitors in BM and peripheral B-cell subsets in spleen, blood and peritoneal cavity. We found similar distribution and cellularity of the analyzed B-cell subsets, with no significant differences between C481S and wild-type mice.

We conducted immunohistochemical analysis of spleens and lymph nodes to determine if the B-cell spatial distribution or the structure of peripheral lymphoid organs remained also unaffected in the C481S model. In spleen, we found maintained follicular structure with well-defined B- and T-lymphocyte areas and unaffected metallophilic macrophage distribution and localization. Lymph nodes from C481S mice were similar to wild-type, with defined central T-cell areas and round B-cell follicles. We further analyzed serum antibody levels and found no significant differences in total IgM, IgG and IgG3. In contrast to the observations in our C481S mouse model, Xid and the corresponding knock-out mice have discontinued MZ macrophage layer, reduced IgM production and very low IgG3 levels (Khan et al., 1995; Ochs and Smith, 1996; Ellmeier et al., 2000). Other non-B-lineage leukocytes and hematopoietic progenitor were also analyzed and we found no significant changes in the C481S animals. Taken together, we demonstrated that mice carrying the C481S substitution have no detectable loss-of-function phenotype and hematopoiesis is overall normal.

We further investigated if the C481S substitution could potentially lead to autoimmune disease, caused by dysregulation of B-cell activation in aged mice. We measured anti-DNA reactivity, evaluated peripheral B-cell subsets and histopathological changes in 16 and 20 months old mice. We did not find any manifestation of autoimmune disease in aged C481S mice. We found

no significant differences in anti-DNA antibodies and in line with the results obtained from young mice, most of the analyzed B-cell subsets from C481S remain similar to the corresponding wild-type subsets. No significant changes were observed when histopathological analysis was carried out for heart, kidney, liver and lung.

Reversible, non-covalent BTKis are a new generation of selective molecules designed to overcome resistance caused by the C481S substitution (Furman et al., 2014; Johnson et al., 2016; Woyach et al., 2017). In order to evaluate if the C481S substitution confers resistance, we performed *in vitro* and *in vivo* inhibition by using the reversible (RN486) and irreversible (ibrutinib, acalabrutinib and zanubrutinib) BTKis.

We measured BTK´s catalytic activity by phosphorylation of Y223-BTK/Y753-PLCγ2 and showed that *in vitro* BTK-inhibition by ibrutinib but not RN486 was affected by the C481S substitution. Ibrutinib blocked phosphorylation of both Y223-BTK and Y753-PLCγ2 in B-lymphocytes from wild-type but minimally from C481S. This finding was confirmed by *in vivo* experiments. When mice were given ibrutinib solubilized in drinking water, BTK´s catalytic activity was inhibited only in wild-type mice.

BTK not only plays a crucial role in development, it is also important for the survival of B-cells (Tsukada et al., 1993; Vetrie et al., 1993; Khan et al., 1995; Bestas et al., 2014). Apoptosis is induced in absence of functional BTK or upon BTK-inhibition (Reiff et al., 2018; Lucas and Woyach, 2019; Wang et al., 2019). We conducted viability assay and demonstrated that stimulated B-cells from C481S mice survived treatment with irreversible BTKis (ibrutinib, acalabrutinib and zanubrutinib) even at high concentrations (ibrutinib and zanubrutinib). In contrast, cell death was observed with RN486 regardless the inhibitory concentration.

Expression of the CD25/CD69 B-cell activation markers upon anti-IgM stimulation and BTK-inhibition was also assessed in wild-type and C481S mice. We found that irreversible BTKis weakly inhibit CD25/CD69 expression, whereas it was completely blocked by RN486. On the other hand, expression of both activation markers was drastically reduced in wild-type cells after stimulation regardless of which inhibitor was used. Taken together, results from BTK´s catalytic activity, viability assay and expression of B-cell activation markers, we concluded that B-lymphocytes from C481S mice are resistant to irreversible, but sensitive to reversible BTKis.

The C481S mouse model, is unique in the sense that it allows us to study the off-target adverse events caused by irreversible BTKis, without blocking BTK expression or catalytic activity. This is because any treatment effect is expected to remain upon binding of the inhibitor to kinases other than BTK.

The IL-2-inducible kinase (ITK) belongs to the group of 10 kinases, which have a cysteine residue in the ATP-binding site similar to BTK and it is crucial molecule for the T-cell receptor-mediated activation and proliferation. We studied if ibrutinib would have a BTK-independent effect on anti-CD3 stimulated T-cells, by measuring the expression of CD25/CD69 activation

markers in wild-type and C481S mice. After anti-CD3 stimulation and ibrutinib treatment, we found >50% reduction of CD25/CD69 co-expression (ibrutinib concentration: 0.25 μM) and total lack of CD25 expression (ibrutinib concentration: 1.0 μM) in both $CD4^+$ and $CD8^+$ T-cells, regardless of mouse genotype. This is in agreement with impaired T-cell function reported in ibrutinib-treated patients (Dubovsky et al., 2013) and demonstrates the potential of the C481S knock-in mouse model to study the mechanism underlying off-target associated side effects and identification of new therapeutic targets.

4.2 Paper II

The gatekeeper residue is located in the regulatory spine of the tyrosine kinase domain (Joseph et al., 2010) and plays an important role in the resistance to several tyrosine kinase inhibitors (Smith, 2017a). Gatekeeper variants have been associated with impaired binding of inhibitors and increased ATP-binding affinity (Shah et al., 2002; Jia et al., 2016; Kay and Dehghanian, 2017). In BTK, gatekeeper variants have been detected in treated CLL patients in presence of the C481S substitution (Maddocks et al., 2015) and co-occurrence of gatekeeper and kinase domain variants in *cis* has been reported by using BTK mutagenesis screening (Wang et al., 2019). In this study we evaluated 16, both single and double BTK variants (Table.1) and demonstrated how simultaneous substitutions of the gatekeeper T474 and the C481 residues located at the ATP-binding side, result in super-resistance to three clinically approved BTKis.

Table 1. Substitutions at T474 and C481. From Estupiñán, H. Y., *et al*. (2021). BTK gatekeeper residue variation combined with cysteine 481 substitution causes super-resistance to irreversible inhibitors acalabrutinib, ibrutinib and zanubrutinib. Leukemia, Online ahead of print. doi:10.1038/s41375-021-01123-6

	Substitutions at T474						Substitutions at C481	
Single substitution	*Alanine (A) GCT	Asparagine (N) AAT	Isoleucine (I) ATT	Proline (P) CCT	Serine (S) TCT		Serine (S)	Threonine (T)
Two or three substitutions	Fenilalanine (F) TTC	Glutamic acid (E) GAA	Glutamine (Q) CAG	Leucine (L) CTG	Methionine (M) ATG	Valine (V) GTG	AGC	ACC

In order to evaluate BTK protein expression and catalytic activity, without influence of endogenous BTK, we performed transient transfection of the gatekeeper variants into the non-lymphoid COS-7 cell line. Similar levels of protein expression were observed for all the gatekeeper variants. The catalytic activity, evaluated by Y223-BTK phosphorylation and compared to wild-type, was reduced in T474A/N/P, T474A/C481S and T474S/C481S variants. Double BTK variants T474I/C481S, T474M/C481S and T474M/C481T showed significantly elevated kinase activity. We confirmed also the expression and activity of selected BTK gatekeeper variants in the lymphoid and non-lymphoid cell lines B7.10 and HEK-293T.

We further investigated if irreversible BTK binders are able to block the kinase activity of the gatekeeper variants, initially by performing *in vitro* inhibition assay with ibrutinib in transfected COS-7 and HEK-293T cells. Ibrutinib at 0.5 μM, which is the pharmacological concentration obtained in serum of treated patients (Byrd et al., 2013), blocked Y223-BTK phosphorylation in most of the single variants and unexpectedly also in T474A/C481S and partially in T474S/C481S double variants. The single T474M variant was fully sensitive only when 4 μM ibrutinib concentration was used, whereas double BTK variants T474I/C481S, T474M/C481S and T474M/C481T showed an interesting super-resistance even when ibrutinib was increased more than 120-fold (64 μM) over the physiological concentration. We confirmed the results obtained from COS-7 cells in HEK-293T cells.

Acalabrutinib and zanubrutinib were used to further study if the ibrutinib super-resistant variants, T474I/C481S, T474M/C481S and T474M/C481T, are also resistant to the second generation of irreversible BTKis. We confirmed that BTK's catalytic activity was not completely blocked even at 96 μM of acalabrutinib, and similar to ibrutinib very high concentration of zanubrutinib (64 μM) was needed to partially inhibit the variants that are super-resistant to ibrutinib.

The sensitivity of the generated substitutions was further examined by utilizing bioinformatic and structural analysis. We hypothesize that super-resistance emerges in the double variants because the binding interactions with the irreversible inhibitors are lost or modified. Substitutions at C481 in BTK affect the ability of the inhibitor to form a covalent bond reducing its binding to only non-covalent targets, which seems to be lost upon the T474M/I replacements at the gatekeeper residue. On the other hand, the unexpected sensitivity of the double variants T474S/C481S and T474A/C481S could be explained by the fact that T474S/A substitutions results in smaller side chains, making the binding pocket wider and accessible to the inhibitor. Additionally, the serine substitution could retain the polar character of the side chain and the hydrogen binding to the inhibitor.

Non-covalent BTKis bind to the H3 pocket in the kinase domain by forming a network of hydrogen bonds in an orthogonal binding mode (Johnson et al., 2016; Crawford et al., 2018; Wang et al., 2019). Therefore, their binding is not affected by the C481S substitution and compounds such as fenebrutinib has shown *in vitro* inhibitory capacity against single C481S/R and T474I/M BTK variants (Byrd et al., 2018). To evaluate the potential of reversible inhibitors as treatment for the BTK super-resistant variants, we obtained the commercially available RN486, fenebrutinib and CGI-1746 BTKis, and tested them in transfected COS-7 cells. We confirmed that all three compounds have inhibitory capacity and could be suitable for the treatment of patients that carry C481S/T substitutions. However, only RN486, at a clinically relevant concentration, showed potential against the ibrutinib super-resistant variants.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The therapeutic landscape of B-cell malignancies has been revolutionized by the introduction of ibrutinib and it evolves rapidly towards the development of more selective BTKis, and molecules with new binding strategies that overcome resistance. The implementation of combined therapies, such as, BTKis with either anti-CD20 or B-cell lymphoma 2 (BCL2) protein inhibitors, has also been reported. Here, I describe the main contribution of this thesis to the research field.

In the **paper I**, we generated a novel knock-in mouse model for the most common resistance mutation cysteine 481 to serine substitution in the catalytic domain of BTK. In the C481S mouse model, we demonstrated that BTK is not susceptible to the treatment with irreversible BTKis, such us, ibrutinib, acalabrutinib or zanubrutinib. Furthermore, by analyzing the effects of ibrutinib on T-cells, we showed the potential of the C481S mice for studying both adverse events and new therapeutic targets unrelated to inhibition of BTK.

Our next step is to initiate treatment of the C481S mouse model with newly developed and more selective BTKis, such as evobrutinib. In a BTK independent manner, we could evaluate by RNA-seq the effects of evobrutinib and compare these with results obtained from C481S mice treated with ibrutinib. It would be interesting to determinate if there are differences in the RNA-seq data from cardiomyocytes, epithelial and blood cells, since evobrutinib does not inhibit ERBB2/HER2, EGFR, RLK/TXK and ITK kinases.

Additionally, we are interested to generate a new mouse model that develops tumors resistant to BTKis. We have acquired Eμ-TCL1 mice that develop spontaneous CLL and do not have any BTK alteration. We will generate a C481S-BTK/Eμ-TCL1 strain, that we speculate would mimic the same neoplastic resistance found in humans. Studies of irreversible BTKis in this new model would provide information about e.g. the effects of the BTKis in a tumor microenvironment not sensitive to BTK inhibition.

In **paper II**, we showed another potential resistance mechanism, caused by threonine 474 to methionine substitution in the BTK gatekeeper residue, for which BTK´s catalytic activity inhibition requires eight times higher ibrutinib concentration. We demonstrated that simultaneous substitution of the gatekeeper residue T474 and of the C481 inhibitor binding site unexpectedly results in super-resistance to the three clinically approved BTKis acalabrutinib, ibrutinib and zanubrutinib. Additionally, we suggested that three non-covalent BTKis (RN486, fenebrutinib and CGI-1746) could be considered as treatment option for ibrutinib-resistant patients with single BTK mutations and RN486 for the super-resistant variants. However, this needs further investigation.

As a follow up of this project we are already studying the influence of gatekeeper residue substitutions for the resistance to newly developed BTKis.

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