CHAPTER I

1. Introduction and Objective

1.1. Kinases

Kinases are enzymes that catalyse an addition of a phosphate group to substrates, usually proteins. The phosphate generally comes from adenosine triphosphate (ATP). This trans-esterification reaction produces a phosphorylated substrate and ADP. This phosphorylated substrate acts as a biochemical messenger in the signal transduction pathway to activate various cellular functions. Kinases are members of the phosphotransferase family and play a key role in cell proliferation, gene expression, metabolism, motility, membrane transport, and apoptosis, and many other cellular pathways, making them vital to human physiology **[1]**.

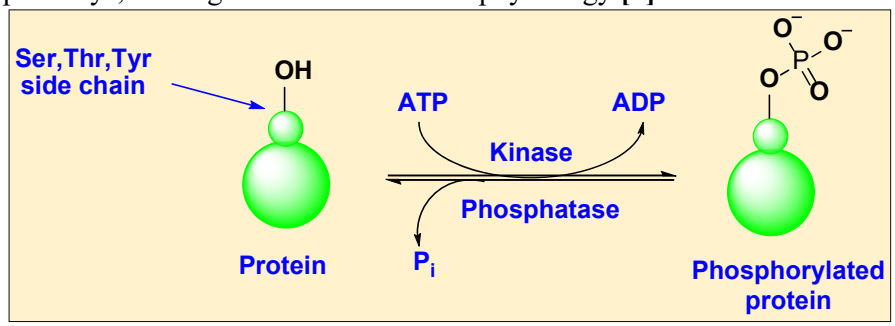


Figure 1. Kinase and phosphatase-catalysed protein regulation.

The human genome contains about 518 protein kinase genes, representing approximately 2% of all human genes. Kinases are classified into three groups based on the type of substrate they act on. Protein kinase, carbohydrate kinase, and lipid kinase.

Protein kinases catalyse the covalent addition of phosphate to target proteins, and this event represents a central mechanism for regulating cellular and enzymatic function.

Eukaryotic organisms possess two general classes of protein kinases, those that transfer phosphate to serine and threonine residues and those transferring phosphate to tyrosine residues. About every part of cellular activity is implicated by protein kinases. It controls metabolism, cell division, motion, and movement, as well as immune and nervous system function and programmed cell death.

According to a review, dysregulation of protein kinases has been linked to over 400 diseases, either directly or indirectly. As a consequence, protein kinases are regarded as one of the most significant drug targets **[2]**. Small molecular compounds that inhibit protein phosphorylation and thus resist activation can be used to target kinases. These small molecule inhibitors reduce kinase gene expression by disrupting ATP-kinase binding, intervening with kinase-protein interactions, and disrupting ATP-kinase binding. Janus kinase (JAK), mitogen-activated protein kinase (MAPK), and Bruton's tyrosine kinase (BTK) are examples of known protein kinase targets.

Carbohydrate kinases play an important role in almost all metabolic pathways, like glycolysis. They are further classified into four classes based on the type of sugar linked substrate: hexokinases, ROK (repressor, open reading frame kinase) kinases, ribo kinases, and GHMP (galactokinase, homoserine kinase, mevalonate, and phosphomevalonate kinase) kinases **[3]**.

Lipid kinases phosphorylate lipids in the cell, both on the plasma membrane as well as on the membranes of the organelles. The addition of phosphate groups can change the reactivity and localization of the lipid and can be used in signal transmission. Phosphatidylinositol-3-kinase (PI3K) and Sphingosine kinases (SK) are types of lipid enzymes, and they regulate a wide variety of cellular functions, including cell growth, proliferation, differentiation, motility, intracellular trafficking, and survival. As a result,

defects in lipid kinase function lead to multiple disease states, including several forms of cancer and diabetes **[4]**.

1.2. Bruton's Tyrosine Kinase (BTK)

Bruton's tyrosine kinase (BTK) is a non-receptor protein tyrosine kinase with a 659-amino acid sequence. The BTK was discovered in 1993 and named after Ogden Bruton, a paediatrician who described X-linked agammaglobulinamia (XLA), in 1952. XLA is a rare syndrome, mainly caused by complete absence of B cell development at the pre-B cell stage, which eventually results in an almost complete absence of B lymphocytes and immunoglobulins in circulation. Patients with XLA have no severe deficiencies in the development of other immune cells, which is consistent with the clinical features that limit B-cell immunity **[5]**. Additionally, patients who are placed on appropriate Ig therapy are pretty healthy, indicating that BTK is superfluous beyond the B cell compartment.

Figure 2. 3D Protein structure of BTK.

BTK originally belonged to the Tec kinase family and is expressed in the majority of hemopoietic cells, primarily B cells and myeloid cells, but not in T cells or plasma cells

[6]. The Tec kinase family also contains bone marrow expressed kinase (BMX), inducible T cell kinase (ITK), TEC, and resting lymphocyte kinase (RLK). Although the protein structure of the Tec kinases is similar to that of the Src family, with an NH_3 domain, SH3 domain, SH2 domain, and a kinase domain, there are still significant differences, such as the complete absence of a myristoylation signal and the COOH terminal tyrosine, both of which are required for Src attachment to the inner surface of the membrane.

An N-terminal pleckstrin homology (PH) domain, a Tec homology domain (TH), a SRC homology 3 (SH3) and SRC homology 2 (SH2) domain, and a C-terminal kinase domain comprise the structures of BTK, ITK, and TEC. The PH domain is a defining aspect of the Tec family of kinases that permits membrane plugging through the use of binding to specific phospholipids. RLK, on either side, lacks a PH domain and instead depends entirely on palmitoylated cysteine to entangle with the membrane **[7]**.

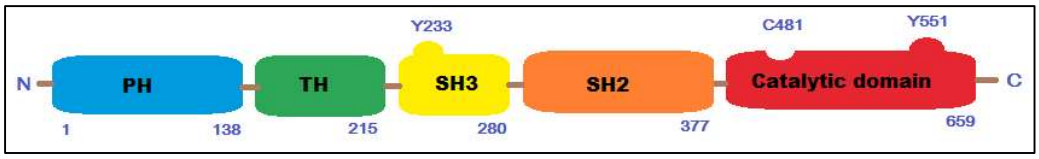


Figure 3. BTK sequence with five distinct domains

1.2.1. PH domain

The PH domain facilitates conditional membrane association, although this is dependent on PI3K activity **[8]**. The unique specificity of the PH domains for phospholipids makes this constrained interaction precisely regulated by individual members of the Tec family of kinases. In comparison to phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] or even phosphatidylinositol-4-phosphate [PI(4)P], the BTK PH domain predominantly binds to phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3] **[9]**. The PH domain runs a range of functions in cellular membranes,

including cytoskeletal organisation, control of intracellular membrane transport, and cellular signalling **[10,11]**.

1.2.2. TH domain

The TH domain has a proline-rich stretch and a "BTK motif," which has just 26 residues and whose function is currently unknown **[12]**. BTK is activated by the proline-rich stretch's interaction with the SH3 domain of the SRC family of kinases **[13]**, while it also regulates its own kinase activity via intramolecular binding to the SH3 domain **[14]**.

1.2.3. SH3 domain

The SH3 domain is essential in the auto regulation of kinase activity, with truncation resulting in constitutive activation of TEC **[15]** and other kinases, namely c-Abl **[16]**. A number of other proteins that adhere to the SH3 region which are necessary for the proper functioning of BTK activity have already been recognised. Wiskott-Aldrich syndrome protein (WASP) **[17]** and VAV **[18]** are two such examples.

Y223, an auto phosphorylation region in the BTK SH3 domain **[19]**, is an early target of the activated kinase Y551. It is believed that Y223 phosphorylation plays a key role in intra- or intermolecular binding interactions.

1.2.4. SH2 domain

Even a few proteins are bound by the SH2 domain, which causes subsequent phosphorylation and activation. Apparently, some of these proteins also require interaction with the PH domain to get phosphorylated **[20]**.

1.2.5. Catalytic domain

The BTK regulatory tyrosine residue Y551 is located in the BTK catalytic domain. The ATP binding region, the catalytic loop, and the allosteric inhibitory sites are also contained in the SH1/TK domain, in addition to the activation loop **[21]**.

1.3. B cell receptor signalling

1.3.1. B cell/lymphocyte

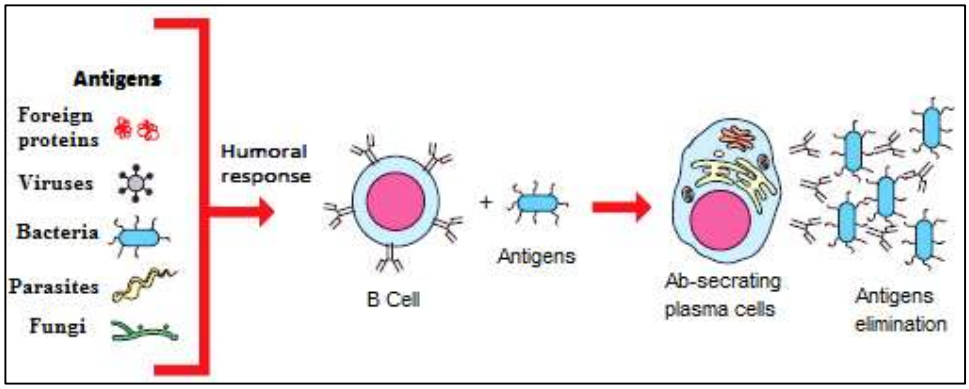


Figure 4. Outline diagram of B-cell function.

B lymphocytes, generally known as B cells, are crucial parts of the adaptive immune response that protects humans from disease **[22]**. B cells continue to be produced in humans throughout their lives, beginning in the foetal liver during intrauterine development and continuing in the bone marrow after birth. Hematopoietic stem cells are primarily responsible for B cell development **[23]**. B-cell development includes all phases of early differentiation prior to antigen interaction, maturation, antigen interaction, and, consequently, antibody synthesis. This procedure resulted in B cells attaining two essential adaptive immunity features: (1) self-versus-non-self-discrimination (the ability of B-cells to recognise foreign antigens instead of self-antigens) and (2) memory (the ability to recall previous antigen contact, resulting in a more effective and faster response during subsequent interaction) **[24]**.

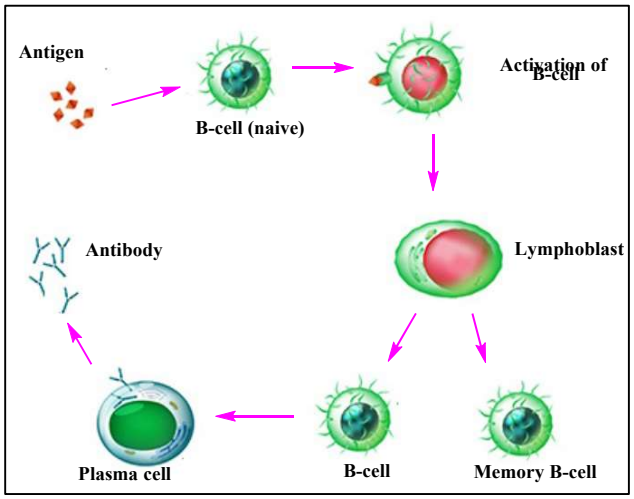


Figure 5. B-cell developmental stages

Continuous differentiation of B cells ensures that B cell repertoires are constantly replenished for limitless antigen recognition. Non-lymphoid cells called stroma make up the matrix of the bone marrow, providing essential molecules for cell production like interleukin-7 (IL-7), cytokines, and adhesion molecules that are critical for B-cell survival and differentiation. Major developmental stages of B cells result in cells that undergo a negative selection process to eliminate self-reactive cells and prevent autoimmunity. Those B cells that survive negative selection will be transmitted through the blood system to peripheral lymphoid organs, where they wait for antigens to react against and ultimately become antibody-secreting cells or plasma cells. However, B cells undergo programmed cell death if they do not encounter antigens **[25]**.

Not all self-antigens are present in the bone marrow, though. In order to prevent B cells from triggering autoimmunity, another tolerance mechanism is in force. Typically, mature B cells require T cells' support to develop antibodies. B cells that are exposed to the antigen and are not accompanied by T helper cells, particular for that antigen will undergo anergy or clonal inactivation. However, a fraction of mature B cells termed as thymus independent B cells (T-independent B cells) have created a new technique for responding to antigens, without the involvement of B cells **[25]**.

Secondary lymphoid organs feature lymphoid follicles, which create a unique environment to concentrate antigen for optimal B cell function. It has follicular dendritic cells that expose antigens to immature B cells. Depending on its location and surrounding environment, secondary lymphoid tissue accumulates antigens from numerous sources: (1) the spleen acquires antigens from the blood, (2) lymphatic nodes acquire antigens captured in the lymphatic system, and (3) mucosa-associated lymphoid tissue (MALT) accumulates antigens from the adjoining mucosal epithelium **[26.27]**.

B cells are in charge of enabling the production of immunoglobulin (Ig), which is tailored against invasive pathogens (antibodies). B cells recognise antigens through secondary cell surface receptors and a membrane-bound B-cell receptor (BCR) **[28]**. B cells can recognise a wide range of structural motifs (epitopes) on antigens due to the extensive sequence and structural diversity of BCR repertoires caused by genetic rearrangement in V(D)J segments, which are responsible for the variable domains (heavy and light chains of BCR).

Antigen stimulation leads stimulated B cells to mature into plasma cells, wherein they release five distinct antibody immunoglobulin classes that start producing substantial amounts of antibody immunoglobulins (IgA, IgG, IgD, IgM, and IgE) **[29]**. After the B cell undergoes mitotic division following activation, a clone of cells with the ability to generate immunoglobulin with identical antigen specificity is formed. Several of these cells will convert into plasma cells. B-cells' initial interaction involving antigens triggers the primary antibody reaction. A section of this clone, indeed, will evolve into memory cells, which will respond promptly to subsequent antigen exposures and trigger a secondary immune response. The secondary immune response is very powerful, occurs really rapidly, and produces IgG rather than IgM. This is the fundamental thought that underlies vaccines and lifetime immunity **[30]**.

B cells play a pivotal role in innate and adaptive immunity, along with stimulating or controlling a wide range of certain other functions required for immune homeostasis. Experimental research revealed that deficient B-cells during murine development cause adverse effects such as congenitally compromised immune systems (e.g., a generalised decrease in number and diversity of T-cells, an absence of Peyer patch organogenesis, and defects within dendritic cells). B-cells are also important for the preservation of the immune system. For instance, b-cells induce immunomodulatory cytokines that govern the actions of immune cells like T-cells and dendritic cells, such as the organogenesis of lymphoid tissue, tissue repair, and the failure of transplanted tissue. Besides this, it has been established that the regulatory B-cell plays a vital role in regulating T-cell-mediated inflammatory reactions by secreting IL-10 **[31, 32]**.

After antigenic stimulation, B cells proliferate rapidly in germinal center, multiplying once every 6 hours. Within lymphoid follicles comprised of follicular dendritic cells (FDCs), the germinal center is a mildly stained site. Somatic hyper mutation is a distinctive activity that takes place once point mutations are initiated into immunoglobulin genes at a high rate, even without repair during the proliferation process **[28]**. Furthermore, Ig class switching plays a major role in B-cell development in the germinal center; even after the completion of B-cell maturation, it contains either IgM or IgD on its surface yet can only release IgM. Class switching is required to facilitate B cells releasing all classes of antibodies in response to variations, such as mucosal infection, where IgA is paramount for eliminating antigens. As a result, mature B cells are exposed to antigens, facilitating their conversion into plasma cells, which produce massive amounts of antibodies and memory cells and respond quickly to antigens in the future **[33]**.

1.3.2. Role of BTK in B cell receptor (BCR) signalling

Peripheral B cells cannot survive without the IgM BCR. B cells exhibit a high rate of apoptosis in the absence of BTK, which is directly linked with a considerably reduced BCR-mediated regulation of the anti-apoptotic protein Bcl-xL. BTK is not required for certain G1 activities, as evidenced by normal cell enlargement and degradation of the cyclin inhibitor $p27^{Kip1}$ in response to anti-IgM stimulation. Due to its failure to trigger cyclin D2 expression, BTK-deficient B cells proceed into the early G1 phase but fail to enter the S phase. Via BTK, the BCR regulates B cell adhesion to vascular cell adhesion molecule-1 (VCAM-1) and fibronectin in addition to B cell survival and proliferation **[34]**.

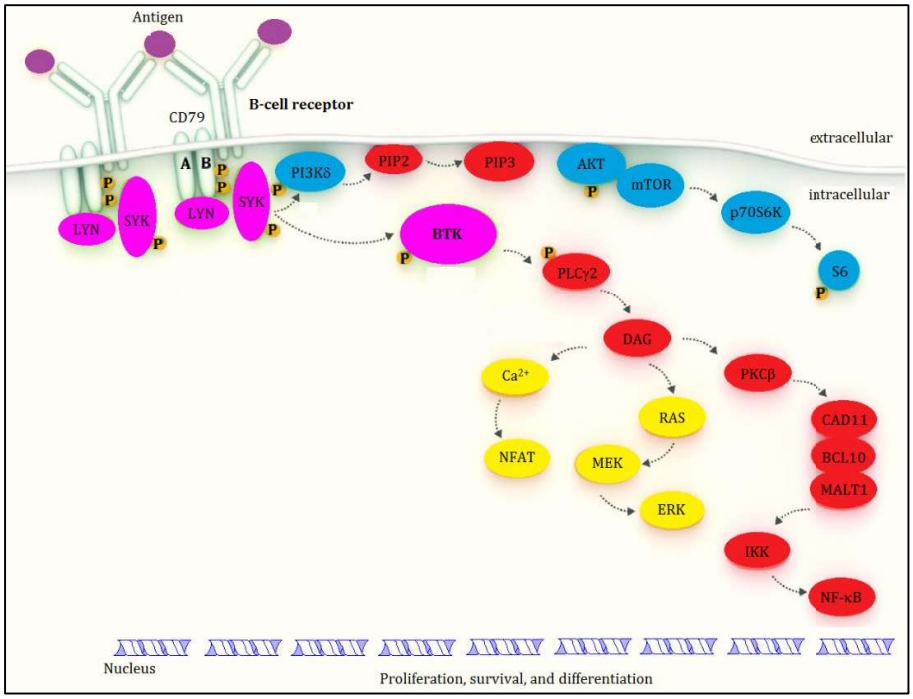


Figure 6. The B-cell receptor signalling pathway

Four families of non-receptor protein tyrosine kinases, including phospholipase C (PLCγ), mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway components, and serine/threonine kinase AKT, are activated through BCR cross-linking **[35]**.

Since the IgM BCR's cytoplasmic domain is so narrow, it does not interface with cells directly and instead interacts with Ig-α/Ig-β (CD79a/CD79b) heterodimers, which are disulfide-linked **[36]**. Immuno receptor Tyrosine-Based Activation Motifs (ITAMs) are found in the cytoplasmic domain of these transmembrane proteins. Antigen-induced stimulation of the BCR enables ITAM to be phosphorylated by Src-family protein tyrosine kinases such as LYN, leading to the formation of docking sites for spleen tyrosine kinase (SYK). Additionally, LYN and SYK phosphorylate tyrosine residues in the cytoplasmic tail of the B-cell co-receptor CD19 and/or the B-cell PI3K adaptor protein (BCAP), which encourages the recruitment and activation of PI3K and the guanine nucleotide exchange factor VAV **[37, 38]**.

Through the activation of RAC1, a GTP ase from the Rho family, VAV further increases the enzymatic activity of PI3K. PIP3 is released after PI3K phosphorylates PIP2, operating as a crucial secondary messenger for triggering downstream pathways. The BTK PH-domain gets mobilised to the plasma membrane as a result of interactions between PIP3 and BTK **[39]**. When SYK is activated, the central B cell-linker molecule SLP65/BLNK is inducted and phosphorylated by the conserved non-ITAM tyrosine residue, Y204, found in Ig-α. PLCγ2 is phosphorylated at Y753 and Y759 with fully activated BTK, which is essential for the lipase function **[40, 41]**.

PIP2 is hydrolysed by activated PLCγ2 to release inositol triphosphate (IP3) and diacylglycerol (DAG). Through calcineurin and calmodulin, IP3 regulates intracellular calcium levels and stimulates nuclear factor of activated T cells (NFAT) transcription. Extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2), including related MAPK receptors such as Jun N-terminal kinase (JNK), p38, and NF-κB pathway factors, are all activated by DAG through the protein kinase Cβ (PKCβ) pathway **[42, 43]**. Hence, the BCR and NF-κB activation are connected by BTK.

Another crucial terminal step in the BCR signalling pathway is activated further upstream. Similar to BTK, PIP3 also interacts with the PH-domain of AKT, leading to its recruitment to the plasma membrane. AKT gets fully activated by phosphorylation at positions T308 and S473 through 3-phosphoinositide-dependent protein kinase-1 (PDK1) and mechanistic target of rapamycin (mTOR) complex 2, respectively **[44]**. Fully activated AKT then returns to the cytoplasm, where it engages in a pro-survival signalling programme involving NFAT, fork head transcription factors (FOXOs), and NF-κB-mediated pathways. Notably, BTK positively regulates AKT phosphorylation **[45]**.

B cells' ability to shift their Ig expression from one isotype to another with a different effector function, such as from IgM to IgG, is imperative in order to perform IGH chain class switching when it has been activated in germinal center (GCs). The IGH variable (V) region does not vary during this cycle, only the constant (C) region varies. It's noteworthy that, in contrast to IgM, the IgG BCR possesses a long cytoplasmic domain with an Ig tail tyrosine (ITT) motif that facilitates signalling **[46]**. IgG BCR-induced calcium mobilisation is accelerated by SYK-mediated ITT phosphorylation, which is followed by the recruitment of BTK through the adaptor protein Grb2. This amplification loop is asserted to be a cell-intrinsic mechanism that allows class-switched memory B cells to quickly activate.

1.3.3. BTK in other signalling pathway

1.3.3.1. Chemokine receptor

These receptors are G-protein-coupled receptors comprising seven transmembrane-spanning domains, along with intracellular heteromeric G-proteins consisting of α, β, and y subunits (Gα, Gβ, and Gy) **[47]**. The chemokine receptors CXCR4 and CXCR5

are expressed on B cells during various points of their development and are essential for trafficking, homing, and homeostasis **[48]**.

Chemokine engagement with its receptor's extracellular domain triggers conformational changes that enable the Gα and Gβy subunits to get detached. Both the Gα and Gβy subunits have the ability to autonomously activate PI3K, leading to the activation of BTK, AKT, and MAPK-based pathways **[49, 50]**. Moreover, by using PH and TH domains, the Gα and Gβy subunits can both directly bind BTK **[50, 51]**. The Gα component has been demonstrated to directly activate BTK activity **[52]**.

BTK is necessary for directing B cells in different lymphoid tissue compartments since it runs downstream of chemokine receptors including CXCR4 and CXCR5. It was already established through adoptive transfer experiments using BTK-deficient B cells that there was decreased *in vivo* migration and lymph node homing **[53]**.

1.3.3.2. Toll-like receptors (TLRs)

Leucine-rich repeats and Toll/interleukin-1 receptor (TIR) domains distinguish these extracellular or intracellular pattern recognition receptors. TLRs, which are expressed in myeloid or B cells, recognise structurally conserved proteins acquired from viruses and bacteria. MYD88 activates interleukin-1 receptor-associated kinase 1 (IRAK1), either on its own or in accordance with TIRAP **[54]**. In the downstream of TLR signaling, BTK interacts with TIR, MYD88, IRAK1, and TIRAP proteins. TLR signalling stimulates transcription factors such as NF-κB, activator protein-1 (AP-1), and interferon regulatory factor 3 (IRF3), which leads to the activation, proliferation, secretion of antibodies, class switch recombination, and production of pro-inflammatory cytokines through B cells **[55-57]**.

1.3.3.3. Fc receptor signalling

BTK functions in the signalling of both inhibitory (ITIM-containing) and activating (ITAM-containing) Fc-receptors, whose balance influences the activation, division, and phagocytosis of myeloid cells **[58-59]**. Following the cross-linking of FcεRI in mast cells, BTK is immediately activated **[60]**. In combination with BCR signalling, SRC-kinases, SYK, PI3K-γ, and BTK are activated once Fc-receptors cross-linking gets activated.

B-cell dysfunction is prevalent in a number of diseases, which include immunodeficiency, autoimmune diseases, and cancer. As previously mentioned, B-cells have the roles of producing antibodies, presenting antigens, regulating the immune system, and maintaining it. Several years of research have compiled evidence that disruption of any of these strictly regulated and controlled pathways could potentially result in autoimmunity, cancer, or other illnesses.

The aberrant production of autoantibodies and the loss of B-cell tolerance are key aspects of autoimmunity. B cell intrinsic dysfunction and the subsequent induction of autoimmunity in the T-cell compartment may be caused by genetic mutations in the B cell lineage. These findings suggest that B cell depletion could be an efficient therapeutic strategy for autoimmune diseases such as B cell malignancies and rheumatoid arthritis **[61, 62]**.

1.4. B cell malignancies/ lymphomas

B cell lymphomas frequently override the mechanisms that facilitate normal B cell differentiation and activation in order to perpetuate their unhindered advancement and survival. The mechanism through which B-cells diversify their repertoire of antibodies can lead to chromosomal translocations and oncogenic mutations, making B cells

particularly susceptible to malignant transformation. Activation-induced cytosine deaminase (AID), myelocytomatosis oncogene cellular homolog (MYC), and BCL6 are protooncogenes that are frequently activated in germinal center B-cell lymphomas by point mutations that seem to arise from "mistargeted" DNA breaks induced by those protooncogenes **[63]**.

Epstein-Barr virus, Human T-cell lymphotropic virus type 1, Helicobacter pylori, Hepatitis C virus, Human herpesvirus 8 (Kaposi sarcoma), Human herpesvirus 6, and Human T-cell lymphotropic virus type 2 are known to activate the protooncogenes that are associated with B cell lymphomas.

Male gender, increasing age, family medical history, cancer history, immunosuppressive agents such as phenytoin and methotrexate, occupational exposure to pesticides, wood dust, epoxy glue, solvents, certain chemicals, UV exposure, nutrient deficiencies, and organ transplants are all potential risk factors for B cell malignancies **[64-69]**.

There are more than 70 types of B-cell lymphoma. the most common types of B-cell lymphoma as follows:

1.4.1. Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL)

The common kind of leukaemia known as chronic lymphocytic leukaemia (CLL) and small lymphocytic lymphoma are alike. Both forms produce small lymphocytes, but SLL induces cancer to grow in the lymph nodes and spleen while CLL primarily impacts the blood and bone marrow. CLL is the most prevalent adult leukemia, with an absolute lymphocyte count well over 5000 per mm^3. The median diagnosis age is 60 years.

SLL covers approximately 4% of NHLs and is connected to lymph node involvement. The common genetic anomalies associated are deletions of 13q14.3, 11q, and 17p, and trisomy 12q **[63, 70]**. Patients may appear to have mild weakness, weight loss, and anorexia, but are generally asymptomatic when diagnosed. In 50–60% of patients, extensive lymphadenopathy and hepatosplenomegaly are often seen. Leukopenia can be observed in patients with SLL and bone marrow dysfunction, and the leukocyte count is usually high.

1.4.2. Follicular lymphoma (FL)

The most prevalent form of indolent NHL is follicular lymphoma (FL). Either men or women are equally affected by it, and it usually develops in middle age. Follicular lymphoma is closely correlated with chromosomal translocations involving BCL2 and is most likely to emerge through germinal center B cells. It is differentiated by a (14; 18) translocation that holds the BCL2 and IGH locus on chromosomes 18 and 14, respectively. The t (14; 18) is involved in up to 90% of follicular lymphomas and enables BCL2 to be overexpressed. Widespread, painless lymphadenopathy is a classic condition of follicular lymphoma. It is quite rare for extranodal systems to be affected, particularly the gastrointestinal tract, central nervous system, or testicles **[63, 70-72]**.

1.4.3. Mantle cell lymphoma (MCL)

A rare lymphoid melanoma is mantle cell lymphoma (MCL). It usually manifests during one's fifth to sixth decade of life and particularly affects men. Often, these mantle cell lymphomas feature an (11;14) translocation involving the IgH locus on chromosome 14 and the cyclin D1 locus on chromosome 11, which translates into cyclin D1 overexpression. Painless lymphadenopathy is the most prevalent symptom. Extranodal sites and symptoms related to spleen involvement (found in about 50% of

cases) are also prominent. Lymphomatous polyposis of the lower gastrointestinal tract is observed in the GIT **[63, 70, 73]**.

1.4.4. Marginal zone lymphomas (MZL)

Marginal zone lymphomas is a category of B-cell malignancy that grows in the lymph nodes, spleen, or extranodal tissues. Numerous cases display symptoms of somatic hypermutation of memory B-cell lineage. The disease begins as a polyclonal immune reaction. Tumors may further create various mutations that end up making their growth and survival antigen-independent, including at the (11; 18), (14; 18), or (1; 14) chromosomal translocations that are often specific and unique for extranodal marginal zone lymphomas **[63, 70]**.

1.4.5. Lymphoplasmacytic lymphoma/ Waldenström macroglobulinemia (WM)

A B-cell neoplasm that affects older adults and typically manifests in the sixth or seventh decade of life, lymphoplasmacytic lymphoma is distinguished by tumour cells that go through final differentiation into plasma cells. Mostly often, the plasma cell portion secretes monoclonal IgM, more often in high concentrations enough to develop into Waldenström macroglobulinemia, a hyperviscosity disorder. Contrary to multiple myeloma, complications associated with the production of free light chains (such as renal failure and amyloidosis) are quite uncommon, and there is no bone deterioration observed.

Nearly 90% of lymphoplasmacytic lymphoma is driven by acquired mutations in myeloid differentiation factor 88 (MYD88) **[74, 75]**. Weakness, fatigue, and loss of weight are among the most common complaints as of now. Hepatomegaly, splenomegaly, and lymphadenopathy adversely impact more than 50% of the patients. Marrow infiltration frequently results in anemia. In around 10% of cases, cold

agglutinins trigger autoimmune hemolysis, the condition known as cryoglobulinemia, which causes symptoms including the Raynaud phenomenon and cold urticaria when macroglobulins precipitate at low temperatures **[63, 70]**.

1.4.6. Burkitt lymphoma (BL)

High-grade B-cell lymphomas, such as Burkitt lymphoma, are composed of medium-sized, highly replicating lymphocytes. They rarely endure a leukemic phase. It is grouped into three categories: (1) African (endemic) Burkitt lymphoma; (2) sporadic (nonendemic) Burkitt lymphoma; and (3) a subclass of aggressive lymphomas that involve HIV-infected individuals.

MYC protein levels elevate as a result of chromosome 8 MYC gene translocations. MYC's translocation partner is very often the IgH locus [t (8; 14)], but it could also be the Ig κ [t (2; 8)] or λ [t (8; 22)] light chain loci. There are usually multiple point mutations evident in the translocated MYC allele. Endemic Burkitt lymphomas have always been inferred to be 100% latently diseased with Epstein-Barr virus (EBV), whereas sporadic and immunodeficiency-associated Burkitt lymphomas are 20% to 40% infected.

Children and young adults tend to be largely infected by endemic and sporadic Burkitt lymphomas. Extranodal sites are involved in many of these tumours. Endemic Burkitt lymphoma unusually impacts the abdominal viscera and normally develops as a mandibular mass. On the other side, spontaneous Burkitt lymphoma mainly affects the peritoneum and ileocecum **[63, 70, 76-78]**.

1.4.7. Diffuse large B-cell lymphoma (DLBCL)

Diffuse large B-cell lymphoma (DLBCL) is one of the major forms of NHL. A substantial masculine high prevalence is evident. The median age of the patients is around 60 years old, although it can also affect adolescents and young children.

DLBCL has a divergent biological composition. The overexpression of BCL6 is one widespread pathogenic consequence. A limit switch in BCL6 is present at chromosome 3q27, and multiple translocations with this switch cause about 30% of DLBCLs. Mutations have also been identified in a variety of similar oncogenes, such as MYC and the anti-apoptotic protein BCL2. Largely, BCL6 rearrangements are missing in tumours with BCL2 rearrangements. MYC coexpression with BCL2 and/or BCL6 is mostly referred to as a "double-hit" or "triple-hit" lymphoma.

DLBCL frequently manifests as a rapidly increasing mass at an extranodal or nodal site. The gastrointestinal tract, skin, bone, brain, and other tissues represent instances of extranodal sites. Involvement of the bone marrow is comparatively rare and occasionally occurs later in the course. A tumoral phenotype hardly ever shows up **[63, 70, 79]**.

1.4.8. Hairy cell leukemia (HCL)

Hairy cell leukaemia (HCL) is a rare but unique B-cell neoplasm that represents around 2% of all B-cell lymphomas. With a median age of 55 and a male-to-female ratio of 5:1, it predominantly affects middle-aged men. Mutations in the signalling genes of the serine/threonine kinase BRAF (B-Raf) cause nearly 90% of cases of hairy cell leukemia. A large proportion of patients present with acute splenomegaly. Lymphadenopathy is uncommon, while hepatomegaly is less frequent. In almost half of the instances, pancytopenia is observed **[63, 70]**.

1.5. Rheumatoid Arthritis (RA)

Rheumatoid arthritis (RA) is a progressive, systemic autoimmune disorder that primarily affects more women than men and is largely seen in older persons. The global prevalence varies by geographic region and accounted about 1% of the population. The synovial joint membrane is severely impacted by RA, which can cause chronic disability, premature mortality, and socioeconomic challenges **[79]**.

The signs and symptoms of symmetrical joint involvement are often arthralgia, inflammation, redness, and even a severely limited degree of motion. Although the actual cause of RA is still unknown, it is evident that environmental factors such as smoking and genetic background are involved.

HLA-DR4 alleles, along with related loci such as protein tyrosine phosphatase, non-receptor type 22 (PTPN22), cytotoxic T-lymphocyte antigen 4 (CTLA4), Fc receptor for IgG (FcγRs) or certain cytokines (TNF, IL-1, IL-10, IL-8), have been closely associated with the disease **[80]**.

A normal, healthy synovial region is composed of cartilage that covers the joint ends of bones, a synovial membrane that is largely formed with fibroblasts and macrophages, and the articular capsule, which is a fibrous, avascular layer that surrounds the synovial cavity. In RA, the synovial membrane is often damaged as a result of extensive immune cell infiltration into joints, causing the creation of destructive cells known as pannus. The synovial membrane spreads, and new blood vessels are produced in the initial stages of RA as a response to the proliferation of synovial cells.

The mobilisation of monocytes, T cells, and B cells from the blood circulation to the synovial cavity is facilitated by angiogenesis, which is associated with altered expression of adhesion molecules on the endothelium and the release of chemokines by

local synovial cells. A conditioned response is enabled by the maturation of monocytes into macrophages, activation, and production of pro-inflammatory cytokines, which in turn stimulate the infiltration of more immune cells. As the disease advances, inflammatory tissue invades cartilage and destroys bone **[81]**.

RA is developed and advanced by various cell types of the innate and adaptive immune systems. The progression of RA is determined by bone deformation analysis using X-rays, rheumatoid factor (RF) analysis, and an IgG Fc autoantibody count in the blood. RF, on the other hand, is likely to be absent during the first years of illness, suggesting that B cells are an integral part of the immune response within the joints but are often not the dominant cells responsible for disease emergence. Immunoassays for the identification of autoantibodies directed against citrullinated proteins have recently gained traction for disease diagnosis. Citrullinated proteins are correlated with the severity of collagen-induced arthritis (CIA) and have been associated with the pathogenesis of RA **[82, 83]**.

1.6. Therapeutic treatments for Rheumatoid arthritis (RA)

Although there's no cure for RA, early treatment and support (including medicine, lifestyle changes, supportive treatments and surgery) can reduce the risk of joint damage and limit the impact of the condition.

In ancient times, RA was treated with bloodletting, leeching, acupressure, needling, moxibustion (the use of heat), cupping, and other techniques. Metal substances, including gold, bismuth, arsenic, and copper, are also used to treat RA. Willow extracts (containing salicin) were used by Hippocrates and Galen to treat the pain of RA and other forms of arthritis. Aspirin (1853), Salicylic acid (1929), Phenylbutazone (1949), and a number of other nonsteroidal anti-inflammatory drugs were also developed.

In 1895, Payne was among the first to propose using quinine to treat RA. Baguall used Chloroquine in 1957, and Hydroxychloroquine is still part of the disease-modifying anti-rheumatic drugs (DMARDs). The therapeutic use of Cortisone in autoimmune disorders, such as RA, was first shown by Philip Hench in 1949. The DMARDs also include the folate antagonist Methotrexate (1950) **[84]**.

There are three general classes of drugs commonly used in the treatment of RA: non-steroidal anti-inflammatory agents (NSAIDs), Corticosteroids, and disease-modifying anti-rheumatic drugs (DMARDs).

1.6.1. Non-steroidal anti-inflammatory agents (NSAIDs)

The primary effect of these agents is to reduce acute inflammation, which reduces pain and improves function. However, it should be noted that these painkillers do not control the progression of the disease or prevent the joint destruction. e.g., Ibuprofen, Naproxen sodium, Aspirin, Diclofenac sodium, Celecoxib, Nebumetone, Piroxicam, Indomethazine, Ketoprofen, Salsalate, Sulindac, etc.

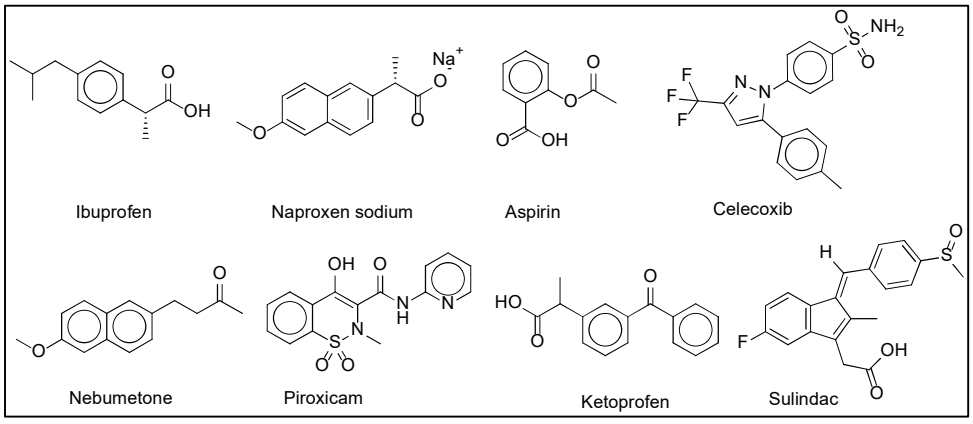


Figure 7. Chemical structure of some well-known NSAIDs

1.6.2. Corticosteroids

Corticosteroids have both anti-inflammatory and immunoregulatory activity. Corticosteroids are useful in early disease as temporary adjunctive therapy while waiting for DMARDs to exert their antiinflammatory effects. e.g., Betamethasone,

Prednisone, Dexamethasone, Cortisone, Hydrocortisone, Methylprednisolone, Prednisolone, etc.

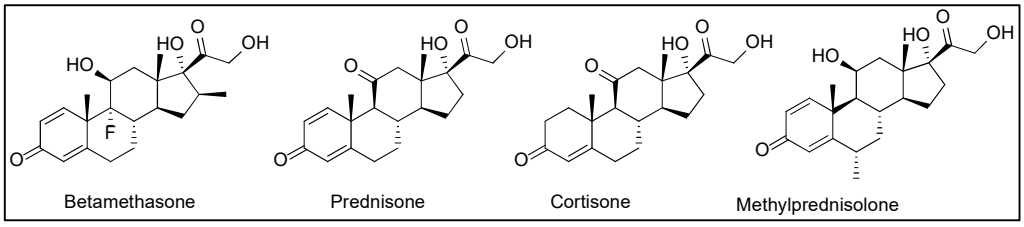


Figure 8. Corticosteroid chemical structures

1.6.3. Disease-modifying anti-rheumatic drugs (DMARDs)

DMARDs are not only used to treat RA pain and/or inflammation, but they can also change the course of the chronic disease and help to suppress some of the damage caused by exacerbations. DMARDs include Methotrexate, Leflunomide, Hydroxychloroquine, and Sulfasalazine. Two subclasses of DMARDs are biologic agents and kinase inhibitors.

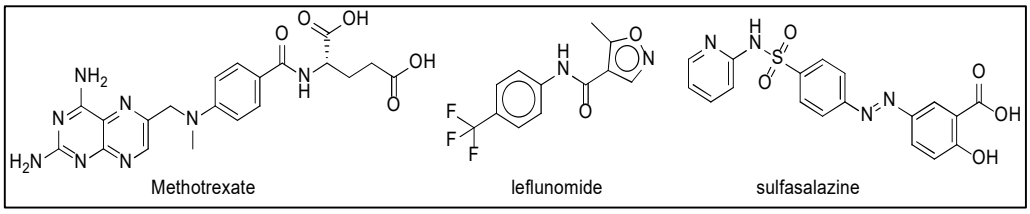


Figure 9. Examples of DMARDs.

1.6.4. Biologic agents

Biological drugs are proteins manufactured using recombinant DNA technology. They are immunosuppressants that target and block the action of cells or chemicals that enable the immune system to cause inflammation and other symptoms of RA. Biologic drugs like Etanercept, Abatacept, Adalimumab, Anakinra, Certolizumab, Tocilizumab, Rituximab, Infliximab, and Golimumab are prescribed for RA treatment.

1.6.5. Kinase inhibitors

Kinase inhibitors are the newest class of drugs used to treat RA. They work by blocking enzymes that are involved in stimulating immune responses in stem cells or other cells,

which contribute to the progression of RA. Tofacitinib, Baricitinib (JAK inhibitors), and Fostamatinib (SYK inhibitors) are approved by drug agencies **[85]**, and some BTK inhibitors are also under clinical development, such as Evobrutinib, Spebrutinib, Acalabrutinib, and Fenebrutinib, for the treatment of RA **[86]**.

1.7. Therapeutic treatments for B cell lymphoma (BCL)

The B-cell lymphoma is staged according to its progression from 1 to 4, with 4 being the most advanced stage. The appropriate treatment regimen for a patient depends on a variety of parameters, particularly the type of BCL, the lymphoma's stage, age, fitness level, and the location of the lymphoma in the body. B-cell lymphoma treatment options typically include the following **[87]**:

1.7.1. Chemotherapy

Chemotherapy is a systemic medication that can be delivered either orally or intravenously. Chemotherapy can be used to cure some aggressive B-cell lymphomas, particularly in cases that are still in their initial stages. DLBCL is a rapidly developing form that can be treated with the CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy regimen. It is termed "R-CHOP" when given in combination with the monoclonal antibody rituximab (Rituxan). It is often treated in several-week cycles. If patients have cardiac issues, then it is not a feasible option as it is risky for the heart. Chemotherapy drugs may cause nausea, fatigue, and hair loss as side effects.

1.7.2. Immunotherapy

Biologic therapies strengthen the immune system in the fight against tumors. Biologic drugs like Rituximab target B-cell-specific genes, which makes it easier for the immune

system to identify and eradicate them. The treatment drives your body to generate new healthy B-cells by decreasing the number of malignant cells. This decreases the likelihood of a cancer recurrence. Radioimmunotherapy drugs, such as Ibritumomab tiuxetan (Zevalin), are composed of monoclonal antibodies that include radioactive isotopes. The drug facilitates the attachment of antibodies to cancer cells, enabling specific radiation delivery. The common side effects of immune therapy are likely low white blood cell counts, fatigue, and infections.

1.7.3. Stem cell transplant

A stem cell transplant means replacing a patient's bone marrow with marrow from a healthy donor. Patients are treated with high-dose chemotherapy or radiation prior to a bone marrow transplant to suppress their immune systems, destroy cancer cells, and make space for the fresh marrow. Patients must be in good enough health to undergo this treatment in order to be eligible. Infections, anaemia, and rejection of new bone marrow could be adverse events.

1.7.4. Targeted Therapy

Target therapies have received a lot of attention from researchers over the last two decades, and as a result, many small molecular compounds have been approved by drug agencies for the treatment of certain types of cancer. Targeted therapies are medications that have been precisely designed to target specific proteins in lymphoma cells. The proteins they target are far more abundant in lymphoma cells than in healthy cells and are directly involved in the survival and proliferation of cancer cells.

Targeted treatments attack lymphoma cells more precisely than chemotherapy. This means they are able to attack lymphoma cells with fewer unwanted effects on healthy cells, leading to fewer side effects. A targeted therapy can be used by itself or in

combination with other treatments, such as traditional or standard chemotherapy, surgery, or radiation therapy.

There are lots of different types of targeted therapy. They all have different functions depending on which proteins they target. The main types that are either already available to treat lymphoma or are in clinical trials for lymphoma are as follows:

1.7.5. Checkpoint inhibitors

Some lymphoma cells could evade detection by the immune system by sticking to a protein on T cells that enabled the T cell not to attack. This protein is inhibited by checkpoint inhibitors. This means that one's T cells could even recognise and attack lymphoma cells. e.g., Nivolumab and Pembrolizumab.

1.7.6. B-cell receptor pathway inhibitors

Chemical messengers are used by all cells to transmit and receive signals. Certain of these signals keep cells alive, whereas others induce them to multiply. B-cell receptor pathway inhibitors prevent these signals in B cells, which proliferate abnormally in B-cell lymphoma. Obstructing the signals can cause B cells to die or stop dividing.

BTK inhibitors, PI3K inhibitors, mTOR inhibitors, and BCL-2 inhibitors are types of B-cell receptor pathway inhibitors. Ibrutinib, Acalabrutinib, and Zanubrutinib are the approved BTK inhibitors. PI3K inhibitors include Idelalisib and Duvelisib. Temsirolimus and Venetoclax are used to inhibit mTOR and BCL-2 enzymes, respectively.

1.7.7. Proteasome inhibitors

Proteasomes are chemicals that break down and recycle proteins inside cells. This maintains protein balance and allows the cell to function properly. It is a natural

function, but it is essential for lymphoma cells because they produce much more protein than healthy cells. Proteasome inhibitors inhibit proteasomes, causing protein blockage in lymphoma cells. The cells can no longer function properly and die. Bortezomib, a proteasome inhibitor, is approved to treat some types of lymphoma.

1.7.8. Immunomodulators

Immunomodulators are medications that improve the way one's immune system responds to lymphoma cells, enabling it to function more efficiently. They also specifically act on lymphoma cells, restricting them from multiplying and activating. Lenalidomide is an example of an immunomodulator drug.

1.7.9. HDAC inhibitors

HDAC inhibitors work by inhibiting the enzyme histone deacetylase. This inhibits enzyme function and could potentially cause tumour cells to die. HDAC inhibitors also enhance the immune system's response to tumour cells. Romidepsin and Vorinostat are available HDAC inhibitors on the market.

As discussed here, targeted therapy has garnered a lot of interest from researchers in recent years because of its unique mode of action and minimal side effects. BTK inhibitor is one of the targeted therapies on which we have focused during current investigation. As per the literature, BTK inhibitors could be a safe and effective target for the treatment of autoimmune disorders such as B-cell lymphoma and rheumatoid arthritis. Hence, we attempted to develop a potent, selective, and orally bioavailable BTK inhibitor. The clinical significance and designing strategies of BTK inhibitors have been outlined in the section that follows.

1.8. BTK inhibitor

A significant role for BTK in B cell growth is illustrated by the X-linked agammaglobulinemia associated with genetic mutations in BTK, which is characterised by a lack of mature B cells and elevated levels of circulating antibody. BTK is engaged in B-cell proliferation and apoptosis in addition to being involved in B-cell survival and differentiation **[88, 89]**.

BTK overexpression has been identified in a number of different B-cell-derived malignancies, including chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), Waldenstrom's macroglobulinemia (WM), and multiple myeloma (MM) **[90-92]**. BTK is also important in some other signalling pathways, such as Fc receptor (FcR), Toll-like receptor (TLR), and chemokine receptor signaling **[93]**.

Recent findings have discovered that BTK is crucial in the development of inflammatory diseases, mainly autoimmune diseases. Loss of self-tolerance, atypical B cell activation, and the eventual formation of autoreactive antibodies are characteristics of autoimmune diseases. Animal studies demonstrate that BTK is essential for determining the threshold for B cell activation and for the BCR signaling-mediated counterselection of autoreactive B cells. Mutant mice overexpressing BTK rapidly captured autoimmune disorders like systemic lupus erythematosus (SLE), which affect many organs. BTK is vital for innate immune cells to release inflammatory cytokines. Eventually, BTK overactivation could cause the occurrence of chronic inflammation or an acute hyperinflammatory condition, making BTK a potential therapeutic target.

Driven by strong preclinical validation, extensive research has been undertaken to discover orally bioavailable novel chemical BTK inhibitors as clinical therapeutic drugs for the treatment of both oncological and autoimmune diseases **[94-100]**. Advanced

BTK inhibitors in preclinical and clinical studies will be discussed in subsequent sections.

1.8.1. BTK inhibitors in preclinical and clinical development

BTK inhibitors can be classified into two groups based on their chemical scaffold, mode of action, and binding mode. (1) Covalent BTK inhibitors are irreversible inhibitors that form a covalent bond with the conserved Cys481 residue in the ATP binding site of the BTK enzyme using the Michael acceptor moiety. (2) Noncovalent BTK inhibitors are reversible inhibitors that bind to a specific pocket in the SH3 domain by stagnant, reversible interactions (e.g., hydrogen bonds or hydrophobic interactions). They specifically access the SH3 pocket of kinase, eventually causing the kinase to become inactive **[101, 102]**. The list of BTK inhibitors are in preclinical and clinical development, as shown in **Table 1**.

Ibrutinib (IBR) is the first generation orally administered BTK inhibitor, which covalently binds to Cys481 of the active adenosine triphosphate (ATP) binding domain of a BTK enzyme **[103]**. IBR was approved by the FDA in 2013 to treat mantle cell lymphoma (MCL) and was subsequently approved for various indications, such as chronic lymphocytic leukaemia (CLL), Waldenstrom's macroglobulinemia (WM), and marginal zone lymphoma **[104]**.

However, IBR not only irreversibly binds to BTK but also induces off-target inhibition of multiple cysteine kinases. Off-target binding of IBR is associated with various adverse effects, such as bleeding, rash, diarrhea, fungal infection, neutropenia, and atrial fibrillation (AF), which may lead to treatment discontinuation. Further, to achieve clinical efficacy, high doses (420–560 mg, q.d.) of IBR is needed due to its poor pharmacokinetic (PK) profile (<3% oral bioavailability (%F) in humans, with variable

exposure) **[105]**. IBR exposure also gets impacted by concomitant CYP3A inhibitors and hepatic impairment, which leads to potential safety concerns **[106]**. Long-term treatment with IBR led to resistance (~60 %), caused by the development of mutated clones (C481S) in the IBR binding site **[107]**.

Acalabrutinib and Zanubrutinib are the second-generation irreversible BTK inhibitors that have been approved by the FDA for the treatment of MCL **[108, 109]**. Acalabrutinib covalently binds to C481 in the BTK with an IC_{50} of 3 nM. It has less off-target binding and a much higher specificity for BTK than IBR. In contrast to IBR, Acalabrutinib does not inhibit the TEC-family kinases (ITK and TXK), ERBB2, and Src-kinases (Src, Lyn, Fyn, Yes, and Lck); however, it inhibits the epidermal growth factor receptor (EGFR), which led to diarrhoea and rashes **[110]**. Acalabrutinib has a shorter half-life than IBR and is given twice daily. In general, cardiovascular events were less common in the Acalabrutinib group compared to IBR **[111]**.

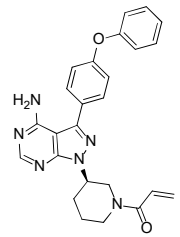
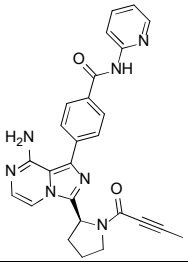
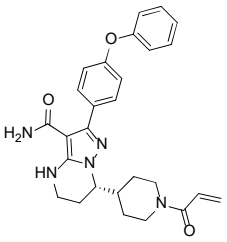
Zanubrutinib exhibits enhanced selectivity for BTK and improved bioavailability relative to IBR. Zanubrutinib demonstrated potent activity and selectivity against BTK over the TEC, EGFR, and Src families. Zanubrutinib showed comparable or improved efficacy and superior safety, with lower incidences of AF, hypertension, diarrhea, and bleeding. These clinical observations are consistent with less off-target inhibition of Zanubrutinib, including ERBB2/HER2 and ERBB4/HER4 (AF), EGFR (diarrhea), and TEC (AF, bleeding).

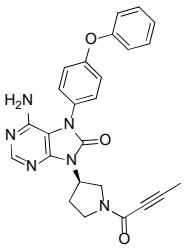
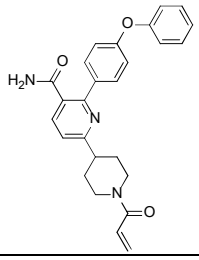
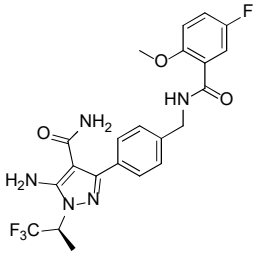
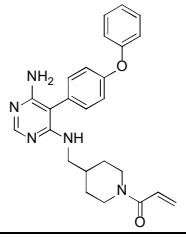
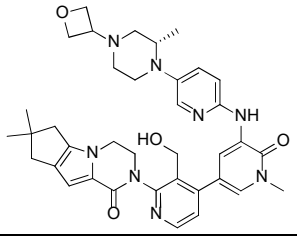
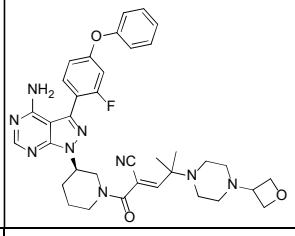
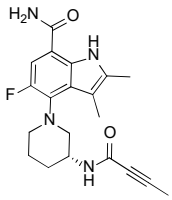
Among the approved BTK inhibitors, Zanubrutinib is less susceptible to modulations of its PK by intrinsic and extrinsic factors, including food, hepatic impairment, and DDI. It is a moderate CYP3A inhibitor. Compared to approved doses of 420 or 560 mg QD for IBR and 100 mg BID for Acalabrutinib, Zanubrutinib doses of 320 mg QD or

160 mg BID provide additional dosing flexibility and increased drug adherence to maximise therapeutic benefits **[112]**.

Other irreversible BTK inhibitors like Tirabrutinib (for BCL), Evobrutinib (for MS), Branebrutinib (for RA, Systemic lupus erythematosus (SLE), and Stevens-Johnson syndrome (SjS)), and Orelabrutinib (for B-cell malignancies and autoimmune diseases) are in clinical development. Reversible BTK inhibitors, Fenebrutinib (for RA and SLE) and Rilzabrutinib (for immune thrombocytopenia (ITP)), are also in clinical development **[100, 113-115]**.

Table 1. Representative chemical structures of covalent and non-covalent BTK inhibitors

BTK inhibitor	Chemical Structure	BTK IC_{50}	Binding mode	Clinical phase	Disease	Developer
Ibrutinib (Imbruvica)		0.5 nM	Covalent	Approved in 2013	MCL, CLL/SLL, WM, MZL, cGVHD	Pharmacyclics
Acalabrutinib (Calquence)		3.1 nM	Covalent	Approved in 2017	MCL, CLL/SLL	Acerta Pharma
Zanubrutinib (Brukinsa)		0.3 nM	Covalent	Approved in 2019	MCL, WM, MZL	BeiGene

Tirabrutinib (Velexbru)		2.2 nM	Covalent	Approved in 2020	CLL, SjS, RA,	Ono Pharma
Orelabrutinib (ICP-022)		4.4 nM	Covalent	Approved in 2020	MCL, CLL/SLL	InnoCare Pharma
Pirtobrutinib (Jaypirca)		5.4 nM	Non-covalent	Approved in 2023	MCL	Eli Lilly
Evobrutinib (MSC-2364447)		8.9 nM	Covalent	Phase-3	MS	Merck
Fenebrutinib (GDC-0853)		0.9 nM	Non-covalent	Phase-2	RA, SLE	Genentech
Rilzabrutinib (PRN1008)		1.5 nM	Non-covalent	Phase-1	ITP	Principia Biopharma
Branebrutinib (BMS-986185)		0.1 nM	Covalent	Phase-1	RA, SLE, SjS	Bristol-Myers Squibb

1.9. Objectives

As previously stated, BTK inhibitor is a clinically validated therapeutic target to treat autoimmune diseases, for which a series of BTK inhibitors have been developed, but they have some drawbacks such as off-target activities, poor pharmacokinetic properties, toxicities, etc. Hence, as part of ongoing research, we aim to design and synthesise a safe, potent, and selective BTK inhibitor for the effective treatment of autoimmune disorders like B-cell malignancies and RA.

To achieve these objectives, we worked according to the following steps:

- Design and synthesis of a new series of compounds as BTK inhibitors.
- Characterization of the chemical structures of the synthesised compounds using NMR, UPLC, CHNS, and ESI-MS analysis.
- The *in vitro* activities of all synthesised compounds evaluated using the BTK enzyme inhibition assay and the TMD8 cell proliferation assay.
- *In vitro* active compounds evaluated for CYP and hERG inhibitory activities.
- *In vivo* pharmacokinetic studies of compounds that are devoid of CYP and hERG will be assessed.
- *In vivo* Pharmacological screening studies of the lead compounds
- Evaluation of anti-tumor activity using the TMD8 xenograft model
- Evaluation of the anti-arthritic efficacy using the collagen-induced arthritis (CIA) mice model
- Evaluation of the safety profile
- Evaluation of Kinase selectivity and Irreversible binding to the BTK enzyme of the lead compounds
- Molecular modelling studies of lead compounds