

# Current Perspectives: Evidence to Date on BTK Inhibitors in the Management of Multiple Sclerosis

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**Abstract:** Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system leading to demyelination and neurodegeneration. Basic and translational studies have shown that B cells and myeloid cells are critical players for the development and course of the disease. Bruton's tyrosine kinase (BTK) is essential for B cell receptor-mediated B cell activation and for normal B cell development and maturation. In addition to its role in B cells, BTK is also involved in several functions of myeloid cells. Although significant number of disease-modifying treatments (DMTs) have been approved for clinical use in MS patients, novel targeted therapies should be studied in refractory patients and patients with progressive forms of the disease. On the basis of its role in B cells and myeloid cells, BTK inhibitors can provide attractive therapeutic benefits for MS. In this article, we review the main effects of BTK inhibitors on different cell types involved in the pathogenesis of MS and summarise recent advances in the development of BTK inhibitors as novel therapeutic approaches in different MS clinical trials. Available data regarding the efficacy and safety of these drugs are described.

**Keywords:** multiple sclerosis, tyrosine kinase inhibitors, Bruton's tyrosine kinase inhibitors, autoimmune diseases, experimental autoimmune encephalomyelitis

## Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) leading to demyelination and neurodegeneration. Although MS aetiology has not been fully elucidated yet, genetic, epidemiological, and pathological studies support the hypothesis that autoimmunity plays a critical role in disease pathogenesis.<sup>1,2</sup>

Classically, CD4<sup>+</sup> T cells have been considered the primary immune drivers in MS. This hypothesis has been supported by two observations: first, MS genetic risk factor mainly resides in the major histocompatibility complex (MHC) class II, which has the ability to perform antigen presentation to CD4<sup>+</sup> T cells and is thus crucial in the development of T-cell central tolerance;<sup>3</sup> second, in experimental autoimmune encephalomyelitis (EAE) –the animal model of MS– the disease can be adoptively transferred to mice through the injection of encephalitogenic CD4<sup>+</sup> T cells.<sup>4</sup> However, basic and translational studies are reshaping this concept. Pathological observations have shown that lymphocytes may be absent in early MS lesions with extensive oligodendrocyte loss and demyelination,<sup>5</sup> whereas normal-appearing white matter (NAWM) may show a profuse infiltration of CD4<sup>+</sup> T cells. Furthermore, in active MS lesions, MHC Class I-restricted CD8<sup>+</sup> T cells (rather than CD4<sup>+</sup> T cells) are known to be clonally expanded.<sup>6,7</sup> Notably, CD4<sup>+</sup> T cell depletion using monoclonal antibodies has rendered no effects on disease activity or course in patients.<sup>8</sup>

T cells do not respond to their antigen in isolation but through their T cell receptor, which must be presented with an antigen by antigen presenting cells (APC) in the context of the MHC molecule. In turn, differentiated T cells can modulate pro-inflammatory or anti-inflammatory differentiation of APCs. Therefore, T cells and APCs interact and can shape each other's immune response. APCs, also commonly known as myeloid cells, represent a heterogeneous population that includes circulating monocytes, macrophages, dendritic cells, tissue resident cells and, in the CNS, microglial cells.<sup>9</sup> In addition, CNS B cell homeostasis and function are relevant to the development and course of

MS.<sup>10,11</sup> Indeed, B cell depletion via the targeting of surface molecule CD20 has proven to be highly effective in the treatment of MS.<sup>12,13</sup> Overall, these data indicate that interactions between T cells, B cells, and myeloid cells promote the immune response in MS.<sup>11,14</sup>

Over the last three decades, numerous disease-modifying treatments (DMTs) have been approved for clinical use, which has impacted the management of MS patients in daily practice and positively influenced their prognosis. Nonetheless, given the heterogeneity of the disease, the use of DMTs may be limited by side effects, comorbidities, or low efficacy in certain groups.<sup>2</sup> For these reasons, the development of new treatment alternatives to curb disease progression remains necessary. In this scenario, research directions are now expanding to change the functional characteristics and differentiation states of immune system components, which may yield an adequate balance between health and disease in response to cues from the local environment.

Transmembrane B cell receptor (BCR) plays a critical role in adaptive immune responses and B cell development and has a unique capacity to recognise an extensive array of antigens due to hypervariable regions in its immunoglobulin heavy and light chains.<sup>15</sup> Antigen recognition triggers specific antibody responses and promotes B cell differentiation into plasma and memory B cells. B cell activation following BCR stimulation is mediated by signalling cascades that involve the activation of membrane-proximal kinases such as spleen tyrosine kinase (SYK), Bruton tyrosine kinase (BTK), and especially PI3K. These kinases have thus become breakthrough therapeutic targets for B cell-malignancies over the past few years, as their aberrant activation drives major hallmarks, including alterations in cellular proliferation, survival, motility, and metabolism.<sup>16,17</sup> Accordingly, inhibitors of SYK,<sup>18</sup> BTK,<sup>19</sup> and PI3K $\delta$ <sup>20</sup> have been developed as alternatives to chemotherapy-based treatments. BTK inhibitor ibrutinib is currently approved for the treatment of patients with chronic lymphocytic leukaemia, mantle cell lymphoma, marginal zone lymphoma, and Waldenstrom macroglobulinemia. However, its off-target safety concerns prevent its use in autoimmune diseases. Second-generation BTK inhibitors have significantly reduced off-target activities for related kinases, improving their safety profiles.<sup>21,22</sup>

BTK also regulates the signalling of other surface receptors, such as chemokine receptors and adhesion molecules which in turn cooperatively regulate the tissue homing of B cells.<sup>22,23</sup> Additionally, BTK has been shown to regulate signalling downstream BCR, FcRs, and Toll-like receptors; these receptors target and modulate the function of other cells in the lesion microenvironment such as myeloid cells, which contain BTK.<sup>24</sup> In other words, as it takes part in numerous pathways and cells other than B cells, BTK is also an attractive target for the treatment of autoimmune diseases such as MS.<sup>2</sup>

In this article, we review the main effects of BTK inhibitors on different cell types involved in the pathogenesis of MS and summarise recent advances in the development of BTK inhibitors as novel therapeutic approaches in different MS clinical trials.

## An Overview on BTK Structure and Signalling

Tyrosine kinases (TKYs) are enzymes mediating tyrosine phosphorylation of diverse molecules that participate in cell proliferation and differentiation, regulate cell growth, and promote cell survival as well as apoptosis.<sup>25–27</sup> TKYs comprise two types of molecules. The first type can induce the autophosphorylation of a receptor or protein substrate, which promotes signal transduction in response to extracellular cues. The second type is intracellular, phosphorylates tyrosine residues on proteins and activates downstream intracellular signalling pathways and, consequently, effector functions.<sup>18</sup> Therefore, given their role in multiple signalling processes, TKYs have emerged as excellent therapeutic targets in different diseases. In this line, BTK –a TKY member of a family of tyrosine kinases expressed in hepatocellular carcinoma (Tec) – is a cytoplasmic protein expressed in many hematopoietic cell types including B cells, mast cells, neutrophils, and myeloid cells. BTK is found in B cells but not in T or NK cells and constitutes an essential component of different BCR pathways after antigen engagement, including the PI3K, MAPK, and NF- $\kappa$ B pathways.<sup>27–29</sup> Indeed, BTK was first described as the molecule responsible for the development of X-linked agammaglobulinemia (XLA), a disease characterised by opportunistic infections resulting from a lack of mature B cells and immunoglobulins.<sup>30</sup>

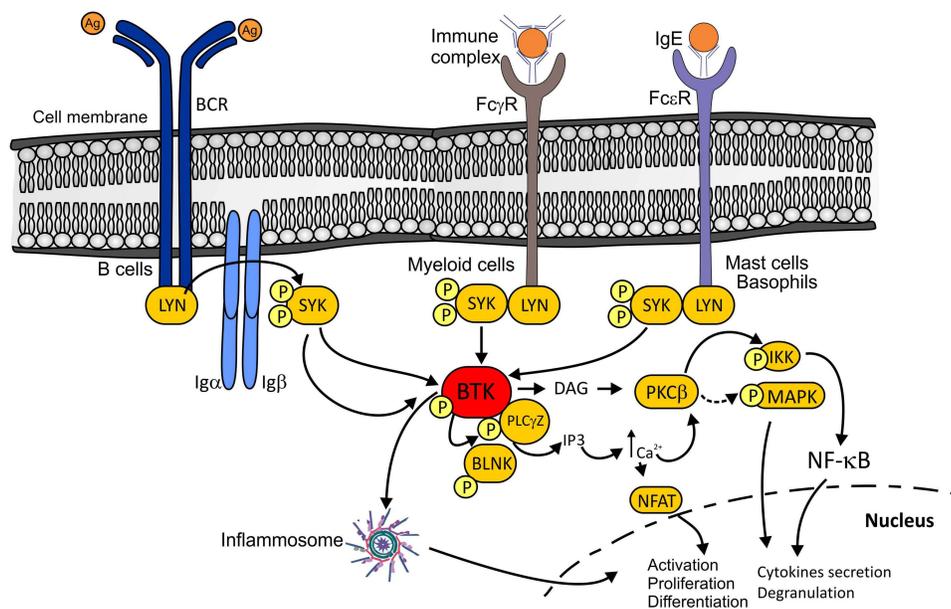
Antigen engagement to the BCR induces its conformational changes, followed by higher levels of kinase activity. BTK is a cytoplasmic protein and is only present on the membrane under recruitment. Once on the cell membrane, BTK can become activated by its interaction with SRC kinases. In turn, this event promotes the recruitment of spleen tyrosine

kinase (SYK) to the membrane, where it is phosphorylated and subsequently phosphorylates BTK. PLC $\gamma$ 2 is then dually phosphorylated by BTK and SYK and subsequently catalyses the cleavage of plasma membrane lipid phosphatidyl 4–5 bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 mediates calcium release from the endoplasmic reticulum, while DAG mediates the activation of protein kinase C $\beta$  (PKC $\beta$ ). Increased calcium levels together with PKC $\beta$  in turn promote the activation of NF $\kappa$ B-, MAPK- and NFAT-dependent pathways, which after nuclear translocation control the transcriptional expression of genes involved in cell proliferation and survival, immunoglobulin class switching, and cytokine secretion.<sup>31,32</sup>

Growing evidence hints at a multifaceted role of BTK in the functions of other cell lineages relevant to the pathogenesis of MS, including (i) signalling through the Fc $\gamma$ R-associated receptor in myeloid cells,<sup>33,34</sup> (ii) the promotion of degranulation and histamine release after the binding of IgE antibodies to the Fc $\epsilon$ R in mast cells and basophils,<sup>35</sup> and (iii) the selective activation of Mac-1 integrin, which enhances neutrophil recruitment during inflammation.<sup>36</sup> Moreover, BTK interacts with Toll like receptors (TLRs), acting as a downstream non-canonical pathway with adapter molecules MAL and MYD88.<sup>37,38</sup> Altogether, these observations have prompted the development of small molecules with the ability to inhibit BTK as potential therapeutics.<sup>25,39,40</sup> A summary of the main BTK signal transduction pathways is shown in Figure 1.

## The Role of BTK in B Cells

Several lines of evidence support the involvement of B cells in the pathogenesis of MS, as B cells have been recognised as infiltrating cells in the brain and spinal cord. Although initially identified as perivascular cuffs but rarely in the parenchyma. B cells and plasma cells were later found in the meninges and perivascular space.<sup>41–44</sup> The presence of abnormally elevated immunoglobulin synthesis rates and a typical oligoclonal band (OCB) pattern of immunoglobulins in cerebrospinal fluid (CSF) are hallmarks of MS, which suggests that immunoglobulins are produced intrathecally.<sup>45</sup> This finding is further supported by somatic hypermutation analysis of B cells and plasma cells, which indicates antigen-



**Figure 1** BTK signal transduction pathways. Bruton's tyrosine kinase (BTK) regulates multiple receptors including B cell receptor (BCR) in B cells, Fc $\gamma$  receptor (Fc $\gamma$ R) in myeloid cells, and Fc $\epsilon$  receptor (Fc $\epsilon$ R) in mast cells and basophils. After activation of these different receptors, they recruit spleen tyrosine kinase (SYK) to the membrane where it is phosphorylated and subsequently phosphorylates BTK. Autophosphorylated BTK is recruited to the plasma membrane and phosphorylates phospholipase C- $\gamma$ 2 (PLC $\gamma$ 2). Activated PLC- $\gamma$ 2 hydrolyses phosphatidyl inositol 4,5-bisphosphate (PIP2), which results in the generation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 upregulates calcium levels and DAG mediates activation of protein kinase C $\beta$  (PKC $\beta$ ). Increased calcium levels, activation of PLC $\gamma$ 2, and PKC $\beta$ , in turn, promote the activation of the NF- $\kappa$ B-, MAPK-, and NFAT-dependent pathways, which control the transcriptional expression of genes involved in cells proliferation, activation, differentiation, cytokines secretion, and degranulation. In addition, BTK is a direct regulator in the activation of the NLRP3 inflammasome, leading to the cleavage and secretion of IL-1 $\beta$ , and IL-18. To facilitate the understanding of the figure, some intermediate pathways have not been drawn.

**Abbreviations:** Ag, antigen; Ig $\alpha$ /Ig $\beta$ , signal transduction moiety (CD79); IKK, I $\kappa$ B kinase; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cells; NF- $\kappa$ B, nuclear factor  $\kappa$ B.

driven B cell activation rather than a random bystander response.<sup>46,47</sup> Therefore, although the antigen specificities of B cells involved in MS remain unclear,<sup>48</sup> antibodies produced can contribute to the development of MS lesions through different mechanisms including opsonisation, complement deposition, and antibody-mediated cytotoxicity.<sup>49,50</sup>

Post-mortem studies indicate that meningeal accumulations of B cells are highly variable in MS. These accumulations can be diffuse or recapitulate features of ectopic lymphoid structures seen in other chronic inflammatory conditions, which is why they are referred to as tertiary lymphoid organs.<sup>7,44,51</sup> Of note, increased expression of molecules associated with sustained B cell activity, such as CXCL13, CXCL10, LT $\alpha$ , interleukin (IL)-6 and IL-10, are present in the meninges and CSF of post-mortem MS tissue, which is consistent with the formation of lymphoid-like structures in the leptomeninges.<sup>52</sup> These structures are more prominent in patients with severe brain inflammation and a rapidly progressing course, which suggests that B cell accumulation contributes to disease worsening.<sup>43,51–54</sup> Indeed, meningeal B cell aggregates have been associated with a gradient of neuronal and oligodendrocyte loss inward from the upper levels of the cortex, associated with microglial activation.<sup>55,56</sup>

CNS-compartmentalised B cells might contribute to MS pathogenesis independently of antibody production. In support of this notion, treatment with anti-CD20 therapies produces a rapid and robust depletion of B cells<sup>12,13</sup> without affecting plasma blasts or plasma cells that express little or no CD20. Given the long half-life of antibodies, it is unlikely that the rapid response to these treatments can be attributed to the removal of pathogenic antibodies.<sup>11</sup> Therefore, memory B cells may possess antibody-independent functions, such as antigen presentation contributing to T cell activation and cytokine production. Recent evidence suggests that one key property of B cells in MS is their ability to recognise antigens through their BCR and to present them via MHC class II to responding T.<sup>57–60</sup> Moreover, memory B cells that express co-stimulatory molecules CD80 and CD86 are found in the CSF of MS patients, which indicates that B cells in the CNS can interact with T cells, promoting the activation and polarisation of CD4+ T cell responses.<sup>61,62</sup> Another important role of B cells is their capacity to produce pro-inflammatory cytokines which can modify the inflammatory environment –increasing T cell and myeloid cell activation– and further contribute to B cell proliferation and differentiation. As compared to those of healthy individuals, memory B cells from MS patients can be activated and produce large amounts of IL-6, tumour necrosis factor (TNF), lymphotoxin- $\alpha$ , and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF).<sup>63–65</sup> The damage dealt by pro-inflammatory cytokines may be balanced by cytokines produced by Breg cells, including IL-10, transforming growth factor  $\beta$  (TGF- $\beta$ ), and IL-35.<sup>66–69</sup>

BTK is essential for BCR-mediated B cell activation and for normal B cell development and maturation.<sup>70</sup> The absence of BTK in humans, for example in XLA, results in a virtual absence of B and plasma cells, as well as in very low levels of circulating immunoglobulins.<sup>71,72</sup> Likewise, animal models involving mutated variants or absence of BTK exhibit an arrest in B cell maturation, reduced B cell numbers and abnormal BCR signalling which fail to produce full BCR engagement into the cell.<sup>73</sup> BTK also takes part in downstream signalling of chemokine receptors,<sup>74,75</sup> cytokine receptors,<sup>76,77</sup> the release of pro-inflammatory cytokines,<sup>70</sup> and in integrin activation,<sup>23,78</sup> with evidence suggesting that these effects are more important in memory B cells.<sup>79</sup> BTK also regulates BCR-induced cytoskeletal remodelling involved in antigen processing and presentation, thereby serving as a common nodal point of regulation for BCR signalling and BCR-mediated antigen-processing pathways.<sup>80,81</sup> In addition to its expected impact on B cell activation, BTK regulates B cell–T cell interactions through a mechanism involving the modulation of B cell mitochondrial respiration (but not glycolysis) and thus affects the ability of B cells to serve as APCs for T cells.<sup>79</sup>

Because BTK plays an important role in B cell activation and differentiation, which in turn mediates the activation of other immune cells also driving MS pathology, BTK fits the profile of a key target for therapeutic intervention beyond B cell depletion.<sup>22</sup>

## The Role of BTK in Myeloid Cells

Myeloid cells, including circulating monocytes, macrophages, dendritic cells, and microglia, have prominent roles in MS pathogenesis.<sup>82,83</sup> These cells function as APCs in the context of MHC molecules and provide co-stimulatory signals for T cell activation and polarisation. Besides antigen presentation, myeloid cells have numerous other effector functions; they can secrete chemo attractive factors and cytokines which may promote either pro-inflammatory or anti-inflammatory T-cell differentiation, participate in the phagocytosis of debris, and generate oxidative stress. In turn, activated T cells can

damage the blood-brain barrier (BBB) and facilitate their transmigration into the CNS by modulating myeloid cells. Therefore, these cell types mutually shape their functional responses, which results in mutual reactivation and the propagation of neuronal injury.<sup>84,85</sup> These different functions highlight the plasticity and functional heterogeneity within these cell populations.<sup>82,83</sup>

Circulating monocytes in MS patients secrete more IL-6, IL-12, and matrix metalloproteinases and have higher expression of co-stimulatory molecule CD86 than in healthy controls, which suggests a pro-inflammatory profile.<sup>86–88</sup> On tissue entry, monocytes differentiate into infiltrating macrophages which can express a broad spectrum of phenotypes with different and opposing functions. Macrophages can be found in the perivascular space, meninges and choroid plexus of MS patients, and they can interact with T cells in the CNS parenchyma after lymphocyte infiltration, in the perivascular space, and in the subarachnoid space of the leptomeninges.<sup>89,90</sup> In the subarachnoid space, macrophages can detect antigens to activate lymphocytes, which subsequently enter the CNS parenchyma.<sup>89</sup> This notion is supported by the fact that the depletion of monocytes and/or macrophages –with liposomes containing clodronate or through CCR knockout– blocks lymphocyte invasion of the CNS parenchyma, reducing or preventing the clinical manifestations of EAE.<sup>91,92</sup>

Pathological studies have demonstrated that activated macrophages and microglial cells are hallmarks of active MS lesions,<sup>93,94</sup> while PET imaging has shown microglial activation in the cortex of MS patients, which corresponds with disability progression.<sup>95</sup> Indeed, microglial activation actively contributes to neuron and oligodendrocyte damage in progressive neurological injury occurring in the subpial region of the cortex and deep grey matter structures such as the thalamus.<sup>96,97</sup> Furthermore, microglia activation is not restricted to lesions but also diffusely present in NAWM and grey matter.<sup>98–100</sup> In NAWM, for example, clustering of activated microglia, so-called microglial nodules, are abundant in areas adjacent to plaques, particularly in patients with progressive MS.<sup>101,102</sup> Transcriptomic studies have revealed multiple microglia activation states within demyelinating lesions, some of which might have a role in inhibiting the pro-inflammatory response and generating a more permissive environment for remyelination.<sup>9</sup> It should be pointed out that activated microglia possess a puzzling array of neuroprotective functions as well, including debris phagocytosis and clearance, growth factor secretion and neuronal circuit shaping.<sup>103,104</sup> Therefore, distinguishing neuroprotective from detrimental phenotypes remains a challenge in the interpretation of microglial function.

In addition to its role in B cells, BTK is also involved in several functions of myeloid cells. BTK expression has been observed in all myeloid cell types, contributing to signalling pathways such as TLRs, cytokine receptors, G-protein coupled receptors, cellular maturation and differentiation and Fc receptor signalling. Most recently, BTK was additionally identified as a direct regulator of the NLRP3 inflammasome through binding to the adaptor ASC domain.<sup>105</sup> However, in contrast to the what is known about BTK in BCR signalling in B cells, the role of BTK in individual myeloid cell populations has not been fully defined yet.<sup>39,106</sup> BTK and Tec have been reported to regulate macrophage-stimulating factor receptor (M-CSFR) signalling, a pathway required for macrophage differentiation, survival, and proliferation.<sup>107</sup> Moreover, macrophages express FcγR on their cell surface, which is crucial for the induction of its phagocytic capacity and cytokine production. Notably, in BTK-deficient mice or upon BTK blockage, the secretion of cytokines mediated by Fc receptors is preferentially affected but their phagocytic capacity remains unaltered.<sup>34,108</sup> Likewise, BTK is critical to TNFα and IL-1β secretion capacity by macrophages through TLR signalling, particularly through TLR4,<sup>109</sup> and to macrophage lineage commitment by regulating the expression of the *Tnfa*, *Il12p40*, *Cxcl10*, *Ccl2*, *Ccl17*, and *Ccl22* genes through the activation of NF-κB or the interferon regulatory factors (IRF) family members.<sup>24</sup> Although dendritic cells (DC) numbers in *Btk*-deficient mice are not affected, these DCs exhibit a more mature phenotype and a stronger in vitro and in vivo T cell-stimulatory ability than wild-type cells. It is thus conceivable that reduced autocrine secretion of IL-10 by BTK-deficient DC and subsequent activation of Stat3 contributes to this DC phenotype.<sup>110</sup>

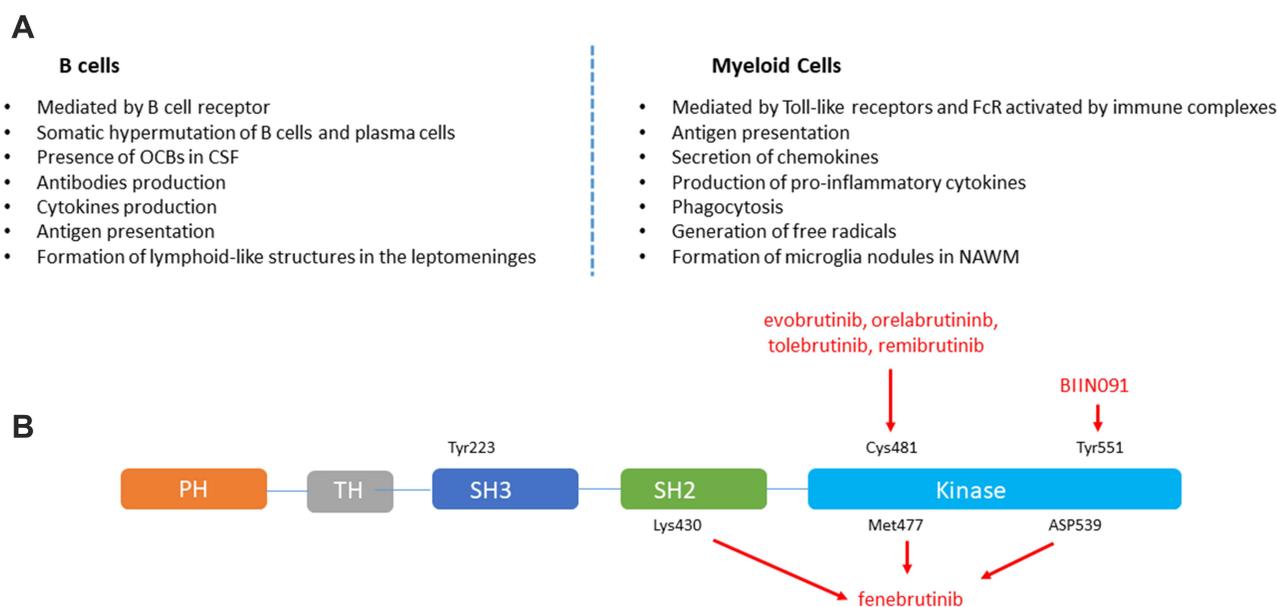
The use of inhibitors of BTK and small interfering RNA (siRNA) against BTK has shown that blockage of BTK activity reduces phagocytosis in rodent microglia and human monocyte-derived macrophages. Inhibition of BTK signalling also reduces microglial uptake of synaptosomes but has only minor impact on other key microglial functions such as migration and cytokine release. Similarly, blockage of BTK function ex vivo in acute brain slices reduces microglial phagocytosis and maintains the number of resting microglia.

Inflammasomes are cytosolic multiprotein complexes which, upon assembly, activate pro-inflammatory caspase-1, responsible for the maturation and secretion of inflammatory cytokines IL-1 $\beta$  and IL-18,<sup>111</sup> and additionally induce pyroptosis.<sup>112</sup> Although inflammasome signalling in the CNS is mainly attributed to microglia, current knowledge of its activation and role in CNS inflammation and disease is only limited and primarily based on in vitro and in vivo studies using transgenic knockout mice which lack the expression of specific inflammasome components throughout the body.<sup>113</sup> Nevertheless, the relevance of inflammasome signalling in microglia and macrophages associated with the edge of lesions has been demonstrated in EAE. Deletion of anti-inflammatory protein A20 in microglia and macrophages of the CNS exacerbates EAE in mice due to the hyperactivation of NLRP3, which results in increased IL-1 $\beta$  secretion and CNS inflammation.<sup>113</sup> On the other hand, the activation of the CNS intrinsic inflammasome has revealed caspase-1-mediated pyroptosis in microglia and oligodendrocytes in the CNS of MS patients and EAE mice.<sup>114</sup> Different studies have identified BTK as a direct regulator of NLRP3 inflammasome activation,<sup>115,116</sup> which is critically required for NLRP3 inflammasome-dependent IL-1 $\beta$  release by murine macrophages.<sup>116</sup> In addition, inflammasome activity is impaired in X-linked immunodeficiency patients, which suggests that a genetic inflammasome deficiency may contribute to immune compromise.<sup>115</sup>

Primary dysfunctions of B cells and myeloid cells in MS are summarised in [Figure 2A](#).

## Targeting BTK for MS Treatment

On the basis of the evidence discussed above, inhibiting BTK may block the activation of different downstream cell signalling pathways associated with MS. Increased BTK activity has been observed in several autoimmune disorders, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).<sup>118,119</sup> For these reasons, the signalling potential of BTK and its targetable nature have been deemed valuable for a wide range of clinical applications. Depending on their mechanisms of action and binding modes, BTK inhibitors can be classified into (i) irreversible inhibitors, which form a covalent bond with the residue Cys481 in the ATP binding site of BTK (the catalytic domain) and exert a powerfully clinical benefit, and (ii) reversible inhibitors, which bind to specific pockets in the targets by weak, reversible forces and thus determine an inactive conformation of BTK. Reversible inhibitors can be easily removed but



**Figure 2 (A)** Primary dysfunction of B cells and myeloid cells in MS and binding sites of the different BTK inhibitory molecules under study. **(B)** Currently available covalent BTK inhibitors bind to cysteine residue 481 in the kinase domain. By contrast, non-covalent BTK inhibitor fenebrutinib forms hydrogen bonds with the lysine 430 residue located in the SH2 region and the methionine 477 and aspartic 539 amino acids found in the kinase domain.

**Notes:** Adapted from Gu D, Tang H, Wu J, Li J, Miao Y. Targeting Bruton tyrosine kinase using non-covalent inhibitors in B cell malignancies. *J Hematol Oncol.* 2021;14(1):40.<sup>117</sup>

lack potency and selectivity.<sup>120</sup> However, despite the stark difference between irreversible and reversible inhibitors, they share many common interacting residues.<sup>121</sup>

Ibrutinib was the first irreversible BTK inhibitor approved for the treatment of B cell malignancies, as well as for graft-versus-host disease. However, ibrutinib inhibits a large number of kinases other than those possessing Cys481-like residues, including intracellular (Tec, Itk, and Blk) and receptor (eg, epidermal growth factor receptors [EGFR] tyrosine kinases, which have been associated with different adverse effects such as cardiac arrhythmias, hemorrhage, hypertension, diarrhea, arthralgia and fungal infections).<sup>25,122</sup> As this off-target activity precludes Ibrutinib evaluation in autoimmune diseases such as MS, alternative second-generation BTK inhibitors which may offer higher selectivity and fewer off-target effects are currently being assessed in clinical trials.<sup>122,123</sup> Although the BTK inhibitors under study in MS are more selective, binding to other non-specific kinases partially persists, with fenebrutinib and orelabrutinib being the most selective for BTK, and tolebrutinib showing most significant binding to other kinases.<sup>124</sup> Furthermore, there are differences in the binding mechanisms (Table 1) and strength of BTK inhibition across molecules. Higher concentrations of evobrutinib are necessary to acquire half of the maximum inhibitory activity (IC50), as compared to tolebrutinib, fenebrutinib, remibrutinib, and BIIB091.<sup>22,125,126</sup> Reversible inhibitors (Table 1) require continuous exposure to the drug over the whole dosing interval to maintain a high degree of target inhibition. Conversely, for irreversible inhibitors, short systemic exposure could be sufficient for sustained inhibition, as the pharmacodynamic effect depends on the turnover of the target–inhibitor adduct rather than on exposure duration.<sup>127</sup>

The efficacy of BTK inhibitors has been demonstrated in EAE.<sup>70,128</sup> Both tyrphostin AG126 and evobrutinib, limit disease severity and have been shown to inhibit B cell activation and maturation and cytokine release, particularly by memory B cells. Furthermore, tyrphostin AG126 reduces Th17 differentiation and microglial activation,<sup>128</sup> while evobrutinib impairs the capacity of B cells to act as antigen-presenting cells for the development of encephalitogenic T cells, thus reducing the number of T-cell infiltrates.<sup>70</sup>

As different from anti-CD20 treatments, which deplete B cells in the periphery, BTK inhibitors can modulate B cells without inducing their depletion. Moreover, given their size, BTK inhibitors can cross the BBB, potentially acting not only on peripheral cells but also on B cells and compartmentalised myeloid cells in the CNS.<sup>129–131</sup> However, these effects have yet to be demonstrated in MS, as evidence has so far only been produced in EAE.<sup>131</sup> In this sense, the differences in immune functions between mice and humans<sup>132</sup> may explain the failure of clinical trials using anti-inflammatory drugs despite positive reports in rodents.<sup>128,132</sup> A summary of the main ongoing clinical trials using BTK inhibitors in MS is shown in Table 1.

## BIIB091

BIIB091 is a novel, potent, highly selective, reversible small-molecule inhibitor of BTK. It has shown potent inhibitory activity against purified BTK protein in an enzymatic assay, demonstrating more than 500-fold selectivity for BTK in relation to all other kinases. BIIB091 selectively and powerfully inhibits B cell and Fc receptor signalling and downstream functions in B cells and myeloid cells. BIIB091 is still in the early stages of development, with its Phase 1 clinical trial (NCT03943056) recently completed but no results published yet. This study evaluated the safety, tolerability, pharmacokinetics, and pharmacodynamics of single and multiple oral doses of BIIB091 in healthy subjects.<sup>125,133</sup>

## Evobrutinib

Evobrutinib is a potent, selective, obligate covalent, BBB-penetrant BTK inhibitor for the treatment of MS.<sup>134</sup> Efficacy and safety results of oral evobrutinib have been recently published in a multi-centre, double-blind, placebo-controlled Phase 2 trial including a total of 267 relapsing MS patients, completed in January 2018 (NCT02975349). Evobrutinib was compared in 3 doses (25 mg once daily, 75 mg once daily and 75 mg twice daily) to placebo or dimethyl fumarate for 24 weeks. The primary outcome was the total number of T1 gadolinium (Gd)-enhancing lesions at 12, 16, 20, and 24 weeks. Secondary outcomes included annualised relapse rate (ARR) and physical disability measured by the Expanded Disability Status Scale.<sup>135</sup> MS patients administered a one daily dose of 75 mg evobrutinib experienced significantly fewer T1 Gd-enhancing lesions at weeks 12 to 24 as compared with placebo-treated ones. Nevertheless, no differences in T1 Gd-enhancing lesions were found with 25 mg once daily or 75 mg twice daily between evobrutinib and placebo

**Table 1** Bruton's Tyrosine Kinase Inhibitors in Clinical Development for MS

Drug and Indication	Pharmacology	Binding Mechanism	Other Kinases (Off-Target Effect)*	Study Details	Stage of Analysis	Clinical Phase	Sponsors and Collaborators
BIIB091 (NCT03943056) Oral Healthy Volunteer	-Molecular weight: 542.64 -Inhibition zone: Tyr 551	-Non-covalent, reversible	AURKA, BMX, CDKL2, LZK, MEK2, PIP5K1C, SCR, TEC, and TIE1. Comparing Kd is >500- fold selective for BTK	-Randomised, vs placebo -Primary purpose: Treatment (Safety, Tolerability, pharmacokinetic and Pharmacodynamic aspects) -Primary outcome: Safety -Secondary outcomes: pharmacokinetic and Pharmacodynamic aspects	Actual enrolment: 64 (completed)	1	Biogen
Evobrutinib (NCT02975349) Oral Relapsing MS	-Molecular weight: 429.51 -Inhibition zone: Kinase domain Cys481 residue.	-Covalent, irreversible.	TEC, BMX (ETK), TXK	-Randomised, vs placebo and dimethyl fumarate -Primary purpose: Treatment (safety and effectiveness) -Primary outcome: new Gd-enhancing lesions on MRI -Secondary outcomes: ARR, disability (EDSS), new T2 lesions and safety	Actual enrolment: 267 (Active, not recruiting) Estimated Study Completion: February 15, 2025	2	EMD Serono and Merck KGaA
Orelabrutinib (NCT04711148) Oral Relapsing MS	-Molecular weight: 427.9 -Inhibition zone: Kinase domain Cys 481 residue.	-Covalent, irreversible.	By KINOMEScan, BTK was the only kinase targeted (> 90% inhibition)	-Randomised, vs placebo -Primary purpose: Treatment (Efficacy, Safety, Tolerability, Pharmacokinetics, and Biological Activity) -Primary outcome: new Gd-enhancing lesions on MRI -Secondary outcomes: ARR, safety and tolerability	Actual enrolment: 160 (Active, recruiting) Estimated Study Completion: March 1, 2024	2	InnoCare
Tolebrutinib (NCT03889639) Oral Relapsing MS	-Molecular weight: 455.51 -Inhibition zone: Kinase domain Cys481 residue.	-Covalent, irreversible.	TEC, ITK, BMX, TXK, EGFR	-Randomised, vs placebo -Primary purpose: Treatment (dose- finding Study) -Primary outcome: new Gd-enhancing lesions on MRI -Secondary outcomes: new T2 lesions, safety and tolerability	Enrolment: 130 (completed)	2b	Sanofi

Evobrutinib (Evolution RMS 1 and 2; NCT04338022 and NCT04338061) Oral Relapsing MS	-Molecular weight: 429.51 -Inhibition zone: Kinase domain Cys481 residue.	-Covalent, irreversible.	TEC, BMX (ETK), TXK	-Randomised, vs placebo and teriflunomide -Primary purpose: Treatment (safety and efficacy) -Primary outcome: AAR -Secondary outcomes: Confirmed disability progression, new Gd-enhancing lesions on MRI, disability (EDSS), new T2 lesions, PROs, Immunoglobulin levels and safety	Actual enrolment: 930 (Active, not recruiting) Estimated Study Completion: April 27, 2022.	3	Merck Healthcare KGaA
Tolebrutinib (GEMINI 1 and 2; NCT04410978/ NCT04410991) Oral Relapsing MS	-Molecular weight: 455.51 -Inhibition zone: Kinase domain Cys481 residue.	-Covalent, irreversible.	TEC, ITK, BMX, TXK, EGFR	-Randomised, vs placebo and teriflunomide -Primary purpose: Treatment (Efficacy and Safety) -Primary outcome: AAR -Secondary outcomes: disability (EDSS), MRI (new or Gd) lesions and safety	Estimated enrolment: 900 (Active, recruiting) Estimated Study Completion: August 2023	3	Sanofi
Tolebrutinib (HERCULES; NCT04411641) Oral Non-relapsing secondary progressive MS	-Molecular weight: 455.51 -Inhibition zone: Kinase domain Cys481 residue.	-Covalent, irreversible.	TEC, ITK, BMX, TXK, EGFR	-Randomised, vs placebo -Primary purpose: Treatment (Efficacy) -Primary outcome: Confirmed Disability Progression -Secondary outcomes: MRI (new, Gd, brain volume loss) lesions, clinical/ cognitive impairment, and safety	Estimated enrolment: 1290 (Active, recruiting) Estimated Study Completion: August 2024	3	Sanofi
Tolebrutinib (PERSEUS; NCT04458051) Oral Primary progressive MS	-Molecular weight: 455.51 -Inhibition zone: Kinase domain Cys481 residue.	-Covalent, irreversible.	TEC, ITK, BMX, TXK, EGFR	-Randomised, vs placebo -Primary purpose: Treatment (Efficacy) -Primary outcome: Confirmed Disability Progression -Secondary outcomes: MRI (new, Gd, brain volume loss) lesions, clinical/ cognitive impairment, and safety	Estimated enrollment:990 (Active, recruiting) Estimated Study Completion: August 2024	3	Sanofi

(Continued)

Table 1 (Continued).

Drug and Indication	Pharmacology	Binding Mechanism	Other Kinases (Off-Target Effect)*	Study Details	Stage of Analysis	Clinical Phase	Sponsors and Collaborators
Fenebrutinib (FENhance 1 and 2; NCT04586023/ NCT04586010) Oral Relapsing MS	-Molecular weight: 664.80 -Inhibition zone: SH2 domain Lys430 residue, and kinase domain Met 477 and Asp539 residues	-Non-covalent, reversible. It does not bind to Cys481. Form hydrogen bonds with Lys430, Met477, and Asp539-	TEC ITK BMX TXK JAK3 EGFR	-Randomised, vs placebo and teriflunomide -Primary purpose: Treatment (Efficacy and Safety) -Primary outcome: AAR -Secondary outcomes: disability (EDSS), MRI (new or Gd) lesions, Total Brain Volume, HRQoL and safety	Estimated enrolment: 736 (Active, recruiting) Estimated Study Completion: November 27, 2025	3	Hoffmann-La Roche
Fenebrutinib (FENtrepid; NCT04544449) Oral Primary progressive MS	-Molecular weight: 664.80 -Inhibition zone: SH2 domain K430 residue, kinase domain M 477 and D539 residues	-Non-covalent, reversible. It does not bind to Cys481. Forms hydrogen bonds with Lys430, Met477, and Asp539	TEC ITK BMX TXK JAK3 EGFR	-Randomised, vs placebo and ocrelizumab -Primary purpose: Treatment (Efficacy and Safety) -Primary outcome: confirmed disability progression -Secondary outcomes: total brain volume, HRQoL, clinical/cognitive impairment and safety	Estimated enrolment: 946 (Active, recruiting) Estimated Study Completion: May 17, 2028	3	Hoffmann-La Roche
Remibrutinib (LOU064) (CLOU064C1230 and CLOU064C12302; NCT05147220) Oral Relapsing MS	-Molecular weight: 507.5 -Inhibition zone: BTK kinase domain Cyst481	-Covalent, irreversible. It does not bind directly to Cys481. Forms hydrogen bonds with Lys430, Met477, and Asp539	TEC inhibitor in vitro, inhibits BTK-dependent platelet activation. Off-target activity measured only against BMX and TEC. Negligible off-target activity against ITK, EGFR, ERB2, ERB3, JAK3	-Randomised, vs placebo and teriflunomide -Primary purpose: Treatment (Efficacy and Safety) -Primary outcome: AAR -Secondary outcomes: disability (EDSS), MRI (new or Gd) lesions, Neurofilament light chain, NEDA, clinical/cognitive impairment, and safety	Estimated enrolment: 800 (Active, recruiting) Estimated Study Completion: November 23, 2029	3	Novartis

**Notes:** \*Overall, the inhibition of other kinases is clinically relevant with IC50 values in the low to high-mid nanomolar range (only those within this range are included here). In general, TEC, JAK and EGFR family member inhibition have been reduced or eliminated in most second-generation BTK inhibitors. The two second-generation BTK inhibitors were designed to bind to cysteine 481 more selectively in the kinase domain and are therefore expected to have fewer off-target effects.

**Abbreviations:** AAR, annualised relapse rate; BMX, bone marrow tyrosine kinase on chromosome X (also known as ETX); EDSS, expanded disability status scale; EGFR, epidermal growth factor receptor; ERBB4, receptor tyrosine-protein kinase erbB-4 (also known as HER4); Gd, gadolinium; HRQoL, health-related quality of life; ITK, interleukin (IL)-2-inducible T-cell kinase; JAK 3, Janus kinase 3; Kd, dissociation constant; NEDA, no evidence of disease activity; MRI, magnetic resonance imaging; MS, multiple sclerosis; PRO, patients reported outcomes; TXK, tyrosine-protein kinase.

treatment at weeks 24 or 48. Data on the open-label extension phase was also recently published for MS patients on 25 mg evobrutinib daily. Neither a dose response nor an effect of evobrutinib on ARR or disability progression were observed in this period.<sup>135</sup> Evobrutinib efficacy was maintained over two years in an open-label Phase II extension study in relapsing MS patients.<sup>136,137</sup> ARR was also maintained stable over 108 weeks. The results published on the 164 patients (61%) who completed at least 132 weeks of treatment in the open-label extension study have shown that the AAR remained low (0.12 relapses per year) in patients assigned to the highest dose of evobrutinib. Interestingly, evobrutinib has shown a beneficial effect on different markers of neuronal damage and chronic tissue loss in MS patients. In this regard, NfLs (a marker of axonal damage) was studied in a sub-analysis with 162 participants (phase 2 trial) and its levels measured since the beginning of the trial. Although high NfL levels can be associated with a more severe disease, including more relapses and brain lesion accumulation over time, treatment with evobrutinib significantly reduced the risk of relapses, regardless of initial NfL levels. Additionally, slowly expanding lesions (SELs) have been related with irreversible brain damage over time. Evobrutinib significantly reduced the size of SELs in a dose-dependent manner when compared to placebo. Other analyses have shown that the dose-dependent effect of evobrutinib on SELs was more robust among subgroups of MS patients with longer disease duration, severe disability, and relapsing MS.

Regarding safety and tolerability, evobrutinib was generally well tolerated and the highest frequency of serious adverse effects was seen with evobrutinib 75 mg twice daily. Nasopharyngitis, a transient increase in aspartate aminotransferase and alanine aminotransferase, and an increase in lipase levels were reported with both 75 mg daily or twice daily, although these adverse effects were not observed in open-label extension patients continuing with evobrutinib. Mild lymphopenia was similar with evobrutinib or placebo, but higher with dimethyl fumarate. Overall, evobrutinib does not appear to be associated with an increased infection risk, including the open-label extension analysis.<sup>137</sup>

As summarised in [Table 1](#), there are currently two identically designed Phase 3 trials underway, EVOLUTION RMS 1 and 2 (NCT04338022 and NCT04338061), to further evaluate evobrutinib vs teriflunomide in relapsing MS patients. These studies have recently finished patient enrolment.

## Orelabrutinib

Orelabrutinib is a highly selective, investigational, oral, obligate covalent, irreversible, BBB-penetrant BTK inhibitor for the treatment of MS. As summarised in [Table 1](#), a randomised, double-blind, placebo-controlled phase 2 study is currently ongoing to evaluate orelabrutinib vs placebo in 160 relapsing MS patients (NCT04711148) and determine efficacy, safety, tolerability, pharmacokinetics, and biological activity. The primary outcome is the cumulative number of new T1 Gd-enhancing lesions (time frame: up to 120 weeks). This study consists of two periods as follows: the core period, where MS patients will be randomly assigned to 1 of 4 treatment groups (placebo, low dose, medium dose, and high dose of orelabrutinib) at a 1:1:1:1 ratio, respectively. The open-label extension period will consist of a single-arm treatment study that will enrol MS patients who have completed the week 24 visit in the core period for continued treatment to generate additional long-term safety and efficacy data. All patients will receive a low dose of orelabrutinib or any other dose as suggested from the core part of the study. Study completion is estimated for March 1, 2024.

## Tolebrutinib

Tolebrutinib is an irreversible, obligate covalent, small molecule, BBB-penetrant BTK inhibitor for the treatment of MS. Efficacy and safety results of oral tolebrutinib have been recently published in a multi-centre, phase 2b, randomised, double-blind, placebo-controlled trial in 130 relapsing MS and relapsing secondary progressive MS patients, completed on January 2, 2020 (NCT03889639). Tolebrutinib was compared in 4 doses (5, 15, 30, or 60 mg once daily, respectively) vs placebo for 12 weeks either before or after placebo administration for 4 weeks. An 85% reduction in the number of new T1 Gd-enhancing lesions and an 89% reduction in the number of new or enlarging T2 lesions were observed upon tolebrutinib treatment as compared to placebo in a dose-dependent manner, the 60-mg dose showing the highest efficacy. Most importantly, brain autopsy samples of secondary progressive MS patients have shown that BTK expression was increased in microglia in and around lesions.

Regarding safety and tolerability, tolebrutinib was generally safe and well tolerated. The most common non-serious adverse effect was headache (in one [3%] out of 33 in the 5 mg group; three [9%] out of 32 in the 15 mg group; one [3%] out of 33 in the 30 mg group; and four [13%] out of 32 in the 60 mg group) followed by chest infections and common cold. No safety-related discontinuations or treatment-related deaths were found.<sup>138</sup>

As summarised in [Table 1](#) two identically designed phase 3 trials are currently ongoing, GEMINI 1 and 2 (NCT04410978 and NCT04410991), to further evaluate tolebrutinib vs teriflunomide in 1800 patients with relapsing MS and active secondary progressive MS. Half of the patients will receive tolebrutinib and the other half teriflunomide for up to 3 years. The primary outcome for both studies is ARR (time frame: up to approximately 36 months). Study completion is estimated for August 2023. In addition, two phase 3, randomised, double-blind, efficacy and safety studies are currently ongoing to compare tolebrutinib to placebo in patients with non-relapsing (non-active) secondary progressive MS (HERCULES; NCT04411641) and primary progressive MS (PERSEUS; NCT04458051). The primary outcome for both studies is time to 6-month clinical disability progression (time frame: up to approximately 48 months for both). Studies completion is estimated for August 2024.

Because of a limited number of cases of drug-induced liver injury in June 2022, the Food and Drug Administration (FDA) placed Phase 3 studies of tolebrutinib in MS and myasthenia gravis on partial clinical hold, and those patients who had been in the trial for fewer than 60 days suspended the study drug. Given that most affected patients had previous concurrent complications predisposing them to drug-induced liver injury, the study protocols were revised to update the monitoring frequency, and enrolment criteria were adjusted to exclude pre-existing risk factors for hepatic dysfunction.

Although multiple health authorities outside the US have permitted the tolebrutinib studies to continue after protocol adjustments that reduce the enrolment of patients with pre-existing liver risk factors, Sanofi has paused recruitment globally for trials still undergoing active recruitment for MS and myasthenia gravis, following the recommendations of the independent data monitoring committee in August 2022. Participants currently receiving tolebrutinib in all studies will continue treatment according to the trial protocols.

## Fenebrutinib

Fenebrutinib is an investigational, oral, non-covalent, reversible, BBB-penetrant BTK inhibitor for the treatment of MS. As summarised in [Table 1](#), two phase 3 trials are currently ongoing to evaluate fenebrutinib. Twin studies FENhance 1 and 2 (NCT04586023 and NCT04586010) are multi-centre, randomised, double-blind, double-dummy, parallel-group studies to assess the efficacy and safety of fenebrutinib compared to teriflunomide in 1468 relapsing MS and active secondary progressive MS patients. The primary outcome for both studies is ARR (time frame: minimum of 96 weeks). Studies completion is estimated for November 27, 2025. Another study is evaluating the efficacy and safety of fenebrutinib compared to ocrelizumab in 946 primary progressive MS patients (FENtrepid; NCT04544449). Participants are being assigned to fenebrutinib or ocrelizumab for 120 weeks. The primary outcome is time to onset of 12-week confirmed disability progression using a composite measure. Study completion is estimated for May 17, 2028.

## Remibrutinib

Remibrutinib is a potent, highly selective, investigational, oral, covalent, irreversible, BBB-penetrant BTK inhibitor for the treatment of MS. As summarised in [Table 1](#), two phase 3 trials are currently ongoing to evaluate remibrutinib. Twin studies CLOU064C1230 and CLOU064C12302 (NCT05147220) are randomised, double-blind, double-dummy, parallel-group studies comparing the efficacy and safety of remibrutinib to teriflunomide in 1600 relapsing MS patients (who will be randomised in a 1:1 ratio), followed by extended treatment with open-label analysis. The primary outcome is ARR (time frame: from baseline, up to 30 months followed by an extension period of up to 5 years). Studies completion is estimated for November 23, 2029.

[Table 1](#) and [Figure 2B](#) summarise the binding sites of the different BTK inhibitors under study in MS.

## Conclusions and Future Perspectives

This review has mainly focused on MS and BTK inhibitors, providing data on newly developed drugs (eg, remibrutinib and orelabrutinib), on-target and off-target effects, and adverse events recently reported by the FDA. However, new

reviews on BTK inhibitors in the management of MS have been published when this review was in preparation.<sup>139–141</sup> It is believed that BTK inhibitors can provide attractive therapeutic benefits for MS, other autoimmune diseases, and haematological malignancies, as they may offer potential advantages over biological components. BTK inhibitors are less likely to elicit anti-drug antibody responses and consequent adverse reactions, are more convenient to administer, and may be more likely to have tissue penetration. Specific inhibition of B cell activation and maturation by BTK inhibitors is a promising new approach for MS treatment. Of note, since the pool of B cells is not depleted, therapy cessation leads to a faster restoration of B cell function, which may render an easier and faster response to unforeseen circumstances such as infections or the need for optimal responses to vaccines. Growing evidence also suggests multiple roles of BTK in cells of the innate immune system, which calls for the development of BTK inhibitors with ability to access the CNS and target microglial activation, a key driver of MS progression. However, there is preliminary evidence that CNS penetrance varies across BTK inhibitors, with tolebrutinib demonstrating greater penetrance than evobrutinib and fenebrutinib.<sup>142</sup> Therefore, whether BTK inhibitors will offer benefits similar to those of biological compounds and whether long-term BTK inhibition will lead to a decline in circulating antibodies remain to be established. Only future research involving head-to-head studies will properly address these questions.

It should be also pointed out that, although ibrutinib provides a breakthrough for therapies for B cell malignancies, the possibility of resistance to treatment due to Cys481 mutations has emerged. In addition, BTK inhibitors might inhibit other tyrosine kinases with structurally related cysteines or act on non-kinase targets of tyrosine kinases (TKIs) not involved in diseases such as MS, which may bring about undesired activity or silent bystander effects which curb their therapeutic use.<sup>143</sup> Furthermore, emerging evidence suggests that BTK function is not limited to catalytic activity and may also serve as structural scaffolds.<sup>27</sup> Although the BTK inhibitors studied in MS differ in selectivity, inhibition strength, binding mechanisms, and CNS penetration, clinical data from long-term clinical trials or real-world data are still unavailable. Therefore, no convincing evidence exists that these factors may determine differences in molecule efficacy and safety. In addition, as different BTK domains may be regarded as therapeutic targets, the success of a particular molecule does not necessarily imply that of other molecules currently under study.

The diversity of immunological pathways targeted by BTKs provides the opportunity to understand human immunology and better design selective BTK inhibitors with minimal off-target effects (Table 1).<sup>22,124,144</sup> Furthermore, identifying the appropriate patient populations is an important consideration given that MS is a heterogeneous disease and, therefore, requires differential treatments.<sup>145</sup> Innovative strategies that could be used to tackle these obstacles include: (i) a multiple-target approach with compounds able to simultaneously target BTKs and focal adhesion kinases;<sup>146</sup> (ii) the association of classical BTKs with other immunotherapies or monoclonal antibodies, as there is strong scientific evidence to consider therapeutics with non-overlapping mechanisms of action; (iii) the use of nanoformulations of BTK inhibitors which are able to reduce toxicity and improve efficacy.<sup>147</sup> Although these are promising routes to explore, they will require the completion of safety studies evaluated through careful trial design to monitor undesired effects.<sup>27</sup>

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