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Bowman-Birk Protease Inhibitor as a Potential Oral Therapy for Multiple Sclerosis

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1. Introduction

1.1. The Bowman-Birk Protease Inhibitor (BBI)

Legume seeds contain different kinds of proteins and protease inhibitors. Serine proteases are a large sub-group of the protease family [1] and they play a role in various pathological conditions such as cancer and thrombotic and inflammatory diseases [2]. Thus they are excellent targets for treatment of many disorders.

Various plant species and, in particular, legumes contain a great number of serine protease inhibitors. The Bowman-Birk protease inhibitor belongs to a family of serine protease inhibitors that has been widely studied for the past 60 years [3, 4].

The soybean-derived Bowman-Birk protease inhibitor (BBI) is a small protein consisting of 71 amino acids and 7 disulfide bonds [4]. BBI is a double-headed serine protease inhibitor, with two functional active sites at opposite sides of the molecule, which inhibits both trypsin and chymotrypsin-like proteases [1,3] (Figure 1). It is a water-soluble protein that is resistant to acidic conditions and proteolytic enzymes [3]. These characteristics make it a good candidate for use as an oral agent for therapeutic purposes.

Crude soybean contains a small amount of BBI and may have components that counter some of the beneficial effects of BBI. Bowman-Birk Inhibitor Concentrate (BBIC) is a soybean extract enriched in BBI [5]. Researchers prefer to use BBIC in their studies because a smaller amount of BBIC contains the proposed dose of BBI compared to crude soybean.

In rodents, BBI is detectable in the blood, tissue and urine after ingestion [6]. Interestingly, BBI can be detected in the central nervous system (CNS) of animals even when the blood-

brain barrier is intact. In human studies, although the BBI level could not be detected in blood after oral BBIC dosing, it could be measured in urine [6].

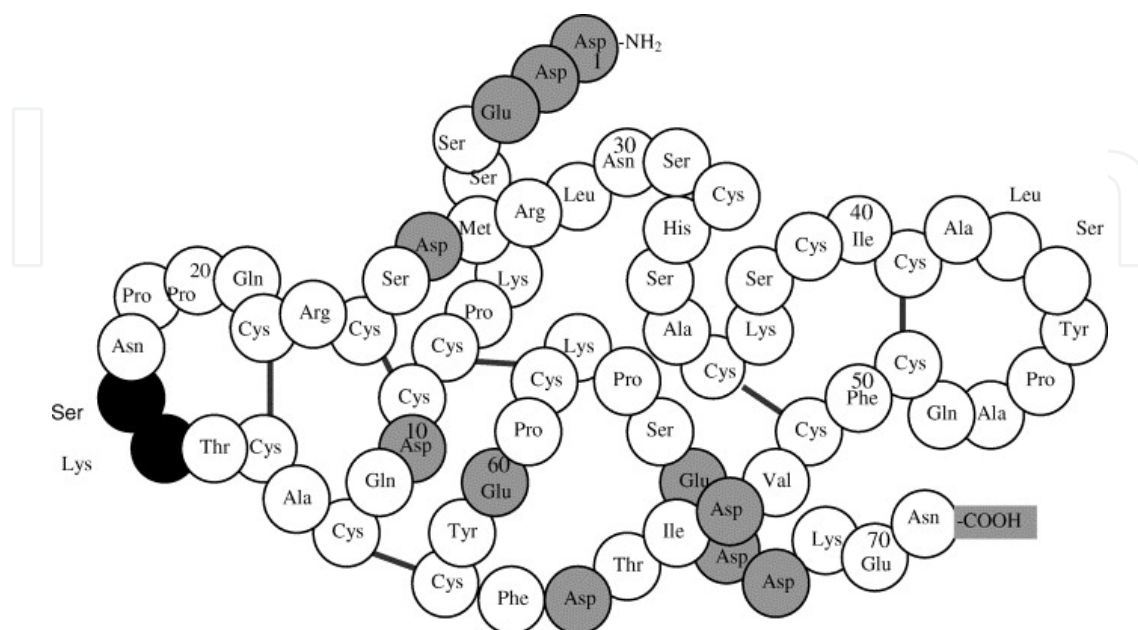


Figure 1. Crystal structure of soybean-derived Bowman-Birk protease inhibitor

The ability of certain serine protease inhibitors to prevent the malignant transformation of cells was shown two decades ago [7, 8]. BBI prevents/suppresses carcinogenesis in a variety of in vitro and in vivo systems [8, 9].

Several human clinical trials to evaluate the effect of BBIC have been completed or are in progress [10-12]. To date, in completed clinical trials, neither toxicity nor neutralizing antibodies against BBIC have been reported in patients receiving BBIC [9].

2. Multiple Sclerosis (MS)

MS is the second cause of disability in young adults and is considered to be a demyelinating disease of the central nervous system (CNS) along with chronic inflammation, demyelination and gliosis [13]. Lesions are characterized by periventricular cuffing and infiltration consisting mainly of T lymphocytes and macrophages, leading to myelin destruction. Recently neuronal degeneration and axonal involvement have also been shown in MS lesions [14]. Current findings therefore raise some doubts about the original assumption that MS is exclusively a white matter disease.

Based on the MS inflammatory phenotype, it has been considered an autoimmune disorder in which peripherally activated myelin-reactive T cells enter the CNS and begin an immunologic cascade that subsequently causes myelin damage.

Activated antigen presenting cells (APCs) and auto-reactive T cells produce pro-inflammatory cytokines, including IL-23, IFN- γ , TNF- α , IL-17, that enhance cell-mediated immunity in the CNS [15-17]. Conversely, other cytokines, such as IL-10, IL-27, IL-4 and TGF- β , play an immunoregulatory role and may be protective in MS [17-20].

Despite extensive research, only a few pharmacotherapeutic agents (e.g., IFN- β , glatiramer acetate, and mitoxantrone) are available, all of which are administered by injection, demonstrate mild to moderate efficacy and have potential side effects [21,22]. A new oral therapeutic agent (Fingolimod) was approved by the FDA and shows potential benefits in MS patients [23].

Recently, in two phase three clinical trials, BG-12 (dimethyl fumarate), a newly proposed oral drug for the treatment of multiple sclerosis, showed a significant reduction in relapse rate and number of MRI lesions in treated patients compared to the placebo group [68, 69].

Development of a new, effective and oral therapy for MS, with fewer side effects, is therefore desirable.

3. Experimental Autoimmune Encephalomyelitis (EAE)

Experimental Autoimmune Encephalomyelitis (EAE) is an autoimmune animal model of MS. Immunization with myelin peptides in different strains of mice induces chronic or relapsing types of the disease, which makes EAE a good tool for studying disease mechanisms and testing therapeutic agents [24]. To date, three of the four therapies currently approved for MS were first tested in this animal model [24].

After immunization with myelin protein, APCs present myelin on the surface of MHC II and produce pro-inflammatory cytokines. Dendritic cell-derived IL-12 and IL-23 lead to development of myelin-specific Th1 and Th17 cells, respectively. Th1 and Th17 cells are the two main culprits in pathogenesis of EAE and MS [25]. Auto-reactive T cells enter the CNS and facilitate recruitment of other immune cells such as monocytes and neutrophils. Accumulation of inflammatory cells within the CNS promotes myelin damage, axonal loss and clinical manifestations in affected animals [24].

Recently it has been shown that dendritic cells are also able to produce another cytokine from the IL-12 family called IL-27. Compared with IL-12 and IL-23, IL-27 elicits different immunoregulatory effects. IL-27 inhibits encephalitogenicity of T cells and suppresses EAE disease [26]. In addition, it stimulates IL-10 production in T cells and induces Tr1 cells [17]. IL-10 is a widely studied immunoregulatory cytokine, which virtually all immune cells are able, in different conditions, to release and which suppresses inflammatory response [27]. IL-10 also plays a significant role in suppression of EAE [28-30].

In general, if a therapeutic agent is able to stimulate IL-10 production and Tr1 cells, it could be an excellent candidate for MS therapy.

4. Proteases in inflammation

Several proteases are associated with the pathogenesis of inflammatory disorders [24, 31]. Proteolytic enzymes are involved in activation and migration of immune cells, cytokine and chemokine activation/inactivation and complement function [32].

Various studies demonstrate that neutrophil serine proteases induce proinflammatory activity of both IL-32 and IL-33 cytokines [33, 34]. They are also able to convert inactive forms of IL-1 and IL-18 to the active form of these cytokines [35]. Cytotoxic T cell-derived proteases called granzymes are also involved in inflammation. Granzymes promote T cell entry into the site of inflammation. In addition, they stimulate B cell proliferation [36].

The complement cascade contains different enzymes that activate each other and proteases that play a role in initiation of the cascade, which results in formation of the membrane attack complex [37].

In general, proteases are involved in all aspects of the immune response and play a significant role in inflammation.

5. Proteases in pathogenesis of EAE and MS

Modulators of neuronal and endogenous proteolysis show a different pattern in spinal cords of EAE rats compared to control animals. This finding indicates higher activity of some proteases in EAE than in control groups, which makes specific proteases good potential biomarkers for disease activity or therapeutic targets in the EAE model and MS [38]. Various types of proteases, including lysosomal proteases and matrix metalloproteinases (MMPs), are highly expressed in MS lesions [24, 39-42]. Serine proteases such as plasmin, cathepsin G, chymase and trypsin activate inert MMP proenzymes to their active forms [24, 41, 42].

GelatinaseB (MMP-9) increases the number of leukocytes entering the site of inflammation and promotes myelin breakdown [39, 43]. Plasmin is a serine protease that mainly participates in the coagulation cascade. It has been demonstrated that plasmin directly induces myelin destruction and demyelination [44].

Levels of gelatinase and tissue plasminogen activator (t-PA) are also increased in MS lesions and in the cerebrospinal fluid (CSF) of active MS patients [46, 47]. Reactive astrocytes and infiltrating lymphocytes, macrophages and microglia express MMP-2, MMP-9 and t-PA in early active MS plaques [24, 41, 45, 47].

6. Anti-inflammatory effects of BBI

BBI suppresses the function of several proteases such as leukocyte elastase, trypsin and human cathepsin G released from human inflammatory cells. [48-50]. Mast cell chymase stimu-

lates migration of lymphocytes and purified T cells, and BBI inhibits this enzyme quite efficiently [49]. In addition, BBI significantly suppresses the chemotactic activity of chymase, thus suppressing lymphocyte migration [51].

Stimulated human polymorphonuclear leukocytes produce reactive oxygen species (superoxide and hydrogen peroxide) that may damage cell membranes by reacting with phospholipids to form peroxides [52]. BBI is able to suppress the production of reactive oxygen species and inhibits their destructive effects [53]. Macrophage-derived proteases and free radicals are also associated with inflammation. BBI down-regulates NO and PGE2 inflammatory pathways in LPS-activated macrophages [54]. Activated macrophages also induce neurotoxicity in the CNS. Anti-inflammatory effects of BBI prevent macrophage-induced neurotoxicity [55].

Serine protease inhibitors can prevent conversion of pro-MMPs to enzymatically active forms [56, 57]. BBI inhibits generation of active MMP-1 and MMP-9 *in vitro*, and BBIC reduces MMP-2 and -9 activity in supernatants of spleen cells [58].

The aforementioned mechanisms may be particularly relevant in the context of the pathogenesis of multiple sclerosis and myelin destruction in the CNS.

BBI may have significant immunomodulatory effects and can be an excellent potential candidate for treatment of inflammatory and autoimmune diseases.

7. BBI and other protease inhibitors in treatment of inflammatory disease

The role of proteases in inflammation has been reviewed in previous sections. Based on the fact that proteases are actively involved in inflammation, they can be a good therapeutic target in suppression of inflammatory response and treatment of inflammatory diseases.

RWJ-355871 is a synthetic protease inhibitor that effectively suppresses allergic inflammatory diseases of the respiratory system [59]. 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF) is another protease inhibitor that attenuates ovalbumin-induced allergic airway inflammation in its animal model [60].

Several studies have reported that protease inhibitors diminish inflammatory response in inflammatory bowel diseases [1]. Nafamostat is a serine protease inhibitor that suppresses dextran sulfate sodium-induced colitis and diminishes inflammatory infiltration in the colon [61]. BBI is able to suppress gland inflammation in the gastrointestinal tract and shows a strong anti-inflammatory effect in the acute colitis model [62]. In addition, in a completed clinical trial [12], BBI demonstrates anti-inflammatory effects and a degree of amelioration of clinical disease and remission rate in patients with ulcerative colitis. We have also shown that administration of oral BBIC significantly inhibits experimental autoimmune neuritis (EAN) in rats [63, 64].

All of the above findings show the potential immunomodulatory and therapeutic effect of BBI in autoimmune diseases.

8. Immunoregulatory effect of BBI in the EAE model

We have shown that oral treatment of BBIC in MBP-induced EAE in rats, reduces disease severity from clinical score 3 (complete hind limb paralysis) to less than 1 (flaccid tail) compared to control animals. In addition, BBIC treatment significantly diminished demyelination in the peripheral nerve tissue of treated animals [58]. We have also shown that both BBI and BBIC suppress clinical and pathologic manifestations of chronic and relapsing EAE in B6 and SJL mice. In addition, the therapeutic effect of oral BBI is dose-dependent, and oral administration of higher amounts of BBI inhibits EAE more efficiently [65] (Figure 1).

BBI treatment also decreased pathogenicity of myelin-reactive T cells and induced milder disease in the adoptively transferred EAE model (unpublished data).

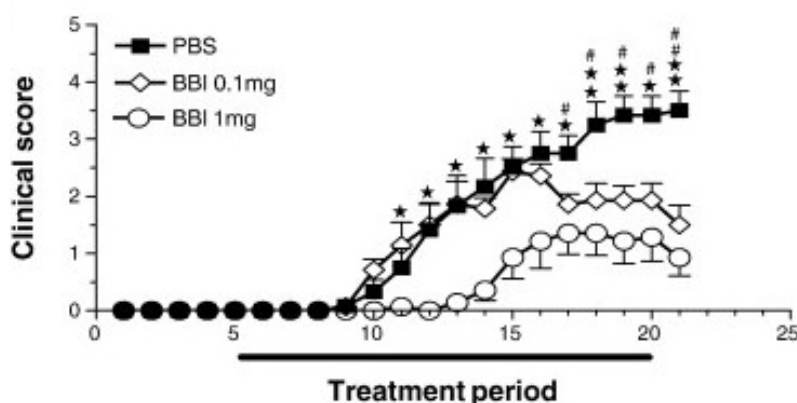


Figure 2. Effect of oral BBI compared to PBS treatment in EAE. Mice that received BBI showed significantly less severe disease compared to control group. The therapeutic effect of BBI is dose-dependent. Elsevier Publications Ltd. has kindly granted us permission to reproduce this figure from Touil et al., 2008.

BBI inhibits invasion of immune cells through the blood-brain barrier (BBB). BBI-treated mice showed dramatically lower numbers of CNS-infiltrating MNCs than control animals [58, 65, 66]. In addition, BBI suppresses generation of active MMP-1 and MMP-9 in vitro, and BBIC reduces MMP-2 and -9 activity in supernatants of spleen cells [58]. Consistent with other findings, BBI decreased migration of splenocytes in Boyden's chamber assay [65].

However, BBI may inhibit release of active MMP-2 and MMP-9 at the blood-brain barrier and prevent immune cell infiltration into the CNS; it might decrease expression of adhesion molecules on immune cells or invasiveness of immune cells, resulting in an altered cytokine pattern of inflammatory cells that hinders their migration from peripheral immune organs to the site of inflammation.

In order to clarify immunoregulatory mechanisms of BBI, the direct effect of BBI on immune cells was evaluated, and it was shown that splenocytes produce a higher amount of IL-10 following BBI treatment [65, 66]. Several reports have demonstrated the immunoregulatory effect of IL-10 in the EAE model of multiple sclerosis. To determine whether the immunomodulatory effect of BBI depends on IL-10, we have compared the therapeutic effect of oral

BBI in EAE in WT and IL-10 KO mice. Although BBI-treated WT mice showed less severe disease, BBI treatment did not affect clinical disease in BBI-treated IL-10 KO mice compared to the control group [66] (Figure 3).

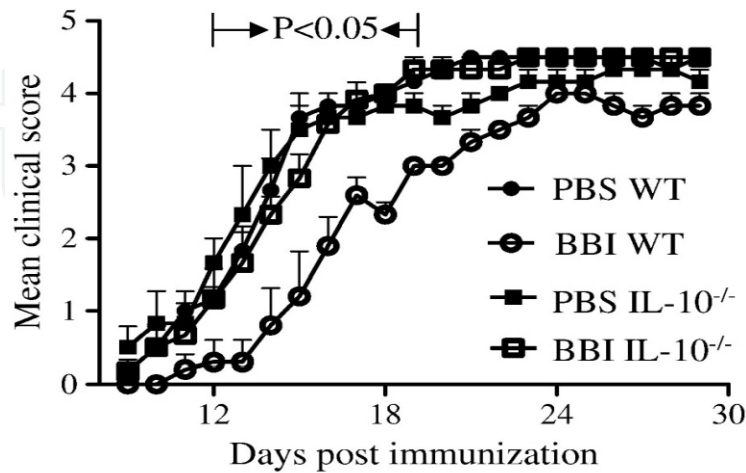


Figure 3. Effect of oral BBI compared to PBS treatment in EAE in WT and IL-10 KO mice. Although BBI-treated WT mice showed significantly less severe disease compared to the control group, there was no significant difference in treated and control IL-10 KO mice. Figure reproduced from Dai et al., 2012 with the kind permission of Elsevier Publications Ltd.

Different types of immune cells can release IL-10 cytokine [27]. However, BBI treatment induces IL-10 mainly in CD4⁺ T cells [66]; it increases IL-10 production in CD8⁺ T cells (unpublished data), demonstrating that BBI has a strong ability to activate IL-10 producing pathways in T cells. Exploring these underlying mechanisms will be a major focus of our future studies.

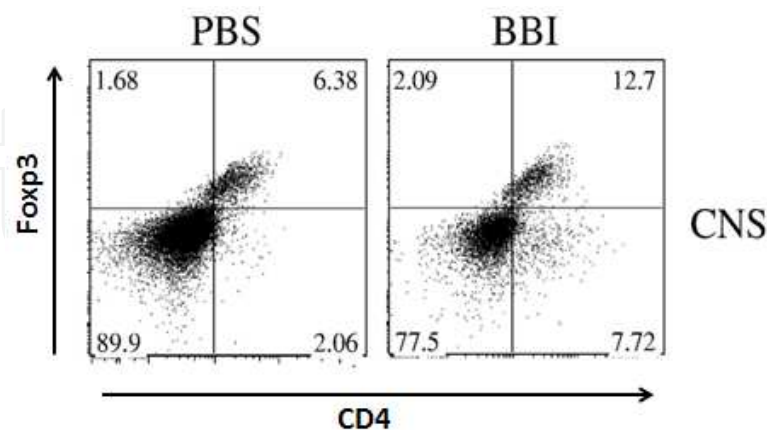


Figure 4. Higher expression of Foxp3⁺ Treg cells in CNS infiltrating cells after oral treatment with BBI, Figure reproduced from Dai et al., 2012 with the kind permission of Elsevier Publications Ltd.

Treg cells are a subgroup of CD4⁺ T cells that expresses the Foxp3⁺ transcription factor. They produce IL-10 in the CNS and can suppress EAE disease [67]. Oral administration of BBI al-

so induces Treg cells in the CNS, which might be one of the underlying mechanisms of the therapeutic effect of BBI in EAE [66] (Figure 4)

BBI also induces IL-10 in other types of effector T cells, and the immunomodulatory effect of BBI might be related to an increase in Tr1 cells. Should this be the case, BBI can be used to induce regulatory T cells and for treatment of autoimmune diseases such as multiple sclerosis.

9. Conclusion

BBI is a soybean-derived serine protease inhibitor. It can be administered orally with several immunomodulatory characteristics and no major side effects. Our observations have shown that BBI dramatically decreases severity of EAE and that its therapeutic effect is mediated through IL-10. In addition, BBI decreases infiltration of inflammatory cells across the BBB and inflammation in the CNS. BBI has potential as a safe and effective oral therapy for multiple sclerosis and other autoimmune diseases.

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