ARTICLE

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The protease inhibitor, Bowman–Birk Inhibitor, suppresses experimental autoimmune encephalomyelitis: a potential oral therapy for multiple sclerosis

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Available treatments for multiple sclerosis (MS) require frequent injections and have significant side effects. Proteases generated during inflammation are involved in the induction of tissue damage during inflammatory demyelination in the central nervous system (CNS). The Bowman–Birk Inhibitor (BBI), a soy-derived protease inhibitor with anti-carcinogenic and anti-inflammatory properties, has been shown to be well tolerated in clinical trials for pre-cancerous conditions, such as oral leukoplakia and the inflammatory disease, ulcerative colitis. We hypothesized that BBI may modulate experimental autoimmune encephalomyelitis (EAE), an animal model of MS. The BBI concentrate (BBIC), a soybean extract enriched in BBI, was administered to myelin basic protein (MBP)-immunized Lewis rats by gastric gavage in different treatment regimens, during the induction or the effector phase of disease. BBIC significantly delayed disease onset and suppressed disease severity, clinically and pathologically, in all treatment protocols. Both *in vitro* and *ex vivo*, BBIC inhibited MBP-specific proliferation of lymph node cells. BBIC reduced the activity of matrix metalloproteinase (MMP)-2 and -9 in spleen cell supernatants and was detected in the CNS of treated rats. BBIC suppresses EAE, it can be administered orally, and it is safe and relatively inexpensive. It may have a therapeutic role in patients with MS. *Multiple Sclerosis* 2006; **12**: 688–697. http://msj.sagepub.com

Key words: experimental autoimmune encephalomyelitis; immunology; matrix metalloproteinase; multiple sclerosis; protease inhibitor; therapies

Introduction

Current therapies for multiple sclerosis (MS) require frequent injections and can have significant side effects [1,2]. Novel treatments that can be administered orally and are well tolerated would be highly advantageous for the treatment of this disease [3].

New therapies for MS are often tested in an animal model of MS, experimental autoimmune encephalomyelitis (EAE) [4]. EAE is induced in susceptible animal strains by immunization with myelin antigens in Freund's adjuvant and is thought to be initiated by myelin antigen-specific CD4+ T cells [5,6]. Autoreactive damage is produced when activated autoreactive CD4+ T cells cross the blood–brain barrier (BBB), recognize myelin antigen on central nervous system (CNS) antigen-presenting cells, produce inflammatory cytokines, and recruit other lympho-mononuclear effector cells by the production of chemokines.

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Protease activity is generated during the inflammatory response leading to demyelination in EAE [7]. Both intracellular and extracellular proteases are thought to contribute to inflammatory damage in EAE through disruption of the BBB and parenchymal injury. Mechanisms of protease-mediated damage in EAE include the generation of matrix metalloproteinases (MMPs) from their inactive precursors [8], intra- and extracellular cleavage of myelin proteins [9,10], and the generation of active complement fractions. MMPs are involved in the induction of BBB breakdown [7,11]. Increased levels of MMP-2, -7, and -9 have been found in the serum, cerebrospinal fluid (CSF), and brain parenchyma of MS patients [12–15]. In addition, MMP inhibitors have been effective in suppressing EAE [16]. Serine proteases inhibitors can block the generation of active MMPs from their precursors [8].

The Bowman–Birk Inhibitor (BBI) is a soybean-derived protein with anti-carcinogenic and anti-inflammatory activity *in vitro* and *in vivo* [17,18]. Functionally, BBI is a double-headed serine protease inhibitor, which inhibits both trypsin- and chymotrypsin-like serine proteases [19]. Chymotrypsin inhibition is preferentially associated with BBI's anti-carcinogenic effects [20]. BBI concentrate (BBIC) is an extract of soybeans enriched in BBI [20].

BBIC has shown a very favorable safety profile in pre-clinical studies and in clinical trials in patients with benign prostatic hyperplasia, pre-cancerous conditions, such as oral leukoplakia, and the inflammatory immune-mediated disease, ulcerative colitis (reviewed in Reference 20).

The goal of the present study was to test the efficacy of orally administered BBIC in EAE. We found significant disease suppression of clinical and histological EAE. These results indicate that BBIC is an excellent candidate for oral therapy in MS patients.

Materials and methods

Rats and EAE induction

Female Lewis rats (6–8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Myelin basic protein (MBP) was purified from guinea pig (GP) spinal cords as described [21]. Rats were immunized with a subcutaneous injection of 100 μg GP-MBP in complete Freund's adjuvant (CFA) containing 2.5 mg/mL of Mycobacterium tuberculosis (H37 RA; Difco, Detroit, MI) in a thoroughly emulsified mixture. Rats were assessed daily in a blind fashion for clinical and neurological signs according to the following scoring system [22]; flaccid tail (1); ataxia (2); partial hind limb paralysis (3); full hind limb paralysis with incontinence (4); death (5). Intermediate scores of 0.5 increments were given to rats with intermediate signs. A cumulative disease severity score was calculated for each rat, adding the daily scores from the day of immunization until sacrifice. Rats were weighed daily by a blind observer using an electronic scale. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

BBIC treatment

BBIC was dissolved in PBS at a concentration of 50 mg/mL. BBIC (200 mg, 4 mL) was administered once or twice daily to each rat by gastric gavage. Control rats received 4 mL of PBS only. Rats were treated from the day of immunization or from day 8, 10, or 12 post-immunization (p.i.) until the day of sacrifice or as indicated. In selected experiments, we compared the *in vivo* activity of PBS, BBIC, and heat-treated BBIC (HT-BBIC) that had been dissolved in PBS and autoclaved twice (at 120°C for 30 minutes) to eliminate serine protease activity [23].

Spinal cord histopathology

Rats were sacrificed at day 16 p.i. and perfused through the left ventricle with 100 mL physiological saline containing 2 U/mL heparin followed by 50 mL of 10% buffered formalin phosphate. The brain and spinal cord were removed and fixed in the same fixative. After 48 hours, the tissue was removed from the fixative and stored in PBS. Tissue was dehydrated in a graded ethanol series, infiltrated with toluene, embedded in paraffin and cut into 5-μm thick sections. CNS sections were stained with Haematoxylin-Eosin (HE) and scored for infiltrates of mononuclear cells as follows [22]: one to five infiltrates per section (1); six to 12 infiltrates per section (2); >12 infiltrates per section (3). For large infiltrates of >150 cells, an additional score of 0.5 was added to the base score.

T-cell proliferation assays

For *in vitro* experiments on the ability of BBIC to suppress myelin antigen-specific lymphocyte proliferative responses, rats were immunized with 100 μg MBP in CFA. On day 16 p.i., rats were sacrificed, injection site draining popliteal and inguinal lymph nodes were removed, and single cell suspensions were prepared in complete RPMI culture medium containing 10% FCS (Hyclone). Cells (10^6/well) were cultured for 60 hours at 37°C in

5% CO_2 in 96-well plates in the presence or absence of 1 μg/mL MBP or 0.5 and 2.5 μg/mL ConA. BBIC was added at different concentrations to test the potential suppressive effect on proliferative responses. Cells were pulsed with $[^3H]$Thymidine (1 μCi/well) for 16 hours and harvested in a Tomtec 96 Mach III harvester onto glass fiber filters. Counts were read with a Beta-counter (Microbeta, Applied Biosystems, Foster City, CA). In *ex-vivo* experiments, lymph node cells were isolated from BBIC- and PBS-treated rats and cultured in 96-well microtiter plates for MBP- and ConA-induced proliferation without BBIC added *in vitro*.

Zymography

Gelatinase activity was tested from supernatants of spleen cells obtained from Lewis rats. Spleen cells were cultured for 72 hours at a concentration of 10^6 cells/mL in 24-well plates in serum-free RPMI culture medium, in the presence or absence of the indicated concentrations of BBIC. Precast 10% Tris–glycine minigels, containing 0.1% gelatin as a substrate, were obtained from Novex (Invitrogen Corp., Carlsbad, CA). Samples were prepared by adding 5 μL of cultured spleen cells supernatant to 5 μL of 2 × Novex Tris–glycine SDS sample buffer for a total volume of 10 μL. Human MMP-2 and -9 (Sigma, St. Louis, MO) (3 μL added to 7 μL of 2 × sample buffer) were used as positive controls. For running buffer, 1 × Tris–glycine from Novex was used. After electrophoresis (at 150 V for 1.5 hours), the gel was washed for 1 hour in 200 mL of 1 × Novex Renaturing Buffer and incubated overnight in 1 × Novex Developing Buffer for maximal proteolysis of the gelatin substrate. Bands of activity were visualized by negative staining using 0.5% Coomasie Brilliant Blue R-250 (Biorad, Hercules, CA) for 15–20 minutes and then de-stained for 8–10 minutes.

Western blot analysis

To determine whether BBIC enters the CNS, we used Western immunoblotting to analyse brain homogenates of rats injected intracardially with 0.1 mg BBIC (dissolved in PBS) or PBS only. BBIC was injected into non-immunized healthy rats and into EAE rats 15 days p.i. After injection (45 minutes), rats were extensively perfused with PBS and brains were harvested, weighed, and snap-frozen in liquid nitrogen. Frozen tissue was ground to a powder using a stainless steel mortar and pestle on dry ice [24]. Each brain was resuspended in 0.1 mg tissue/mL buffer (1 × PBS with 0.5% Na deoxycholate, 0.05% Triton X-100, 5 mM $MgCl_2$ $6H_2O$, 2 μM PMSF, in 1 × PBS) supplemented with 1 Roche Complete Mini protease inhibitor cocktail tablet per 7 mL buffer (Roche Diagnostic Corp., Indianapolis, IN). Suspensions were sonicated three times for 10 seconds on ice using a Misonix sonicator (Farmingdale, NY). Protein precipitation was performed as described [25].

For Western blot analysis, brain extract aliquots or BBIC samples, as positive controls, were electrophoresed on a 12% Novex Tris–glycine gel (Invitrogen) and transferred to a nitrocellulose membrane via a semi-dry transfer apparatus. Detection of the BBI protein was performed using the 5G2 anti-BBI primary antibody, which recognizes both reduced BBI and BBIC [26]. BBI protein was detected using peroxidase conjugated affini-pure goat-anti-mouse IgG (H&L) secondary antibody (1:2000) (Jackson Immuno Research Laboratories, West Grove, PA) and ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

Statistics

The Mann–Whitney *U*-test or the Student's *t*-test for unpaired samples was used for the comparison of clinical scores and proliferative responses. Fisher's exact test was used to compare disease incidence in different treatment groups. All significance tests were two-sided. GraphPad Prism 3.02 (GraphPad Software, Inc., San Diego, CA) and SigmaStat (Systat Software, Inc., Point Richmond, CA) software were used for statistical analysis.

Results

BBIC treatment at the time of induction suppresses clinical EAE

To test the efficacy of BBIC in the suppression of EAE, we first administered BBIC by gastric gavage to MBP-immunized rats from day 0 to 16 p.i. BBIC significantly delayed EAE onset (PBS: 11.3 ± 1.0 days p.i.; BBIC: 13.6 ± 1.2 days p.i.; $P < 0.001$), disease duration (PBS: 5.7 ± 1.1 days; BBIC: 2.8 ± 2.0 days; $P < 0.001$), maximal clinical score (PBS: 3.2 ± 1.5; BBIC: 1.3 ± 0.9; $P < 0.001$), and cumulative score (PBS: 13.3 ± 6.8; BBIC: 3.9 ± 2.9; $P < 0.001$). One representative experiment out of three with similar results is shown in Figure 1.

BBIC treatment during the effector phase of EAE suppresses disease

We tested the efficacy of BBIC in suppressing EAE when administered at the time of the effector phase

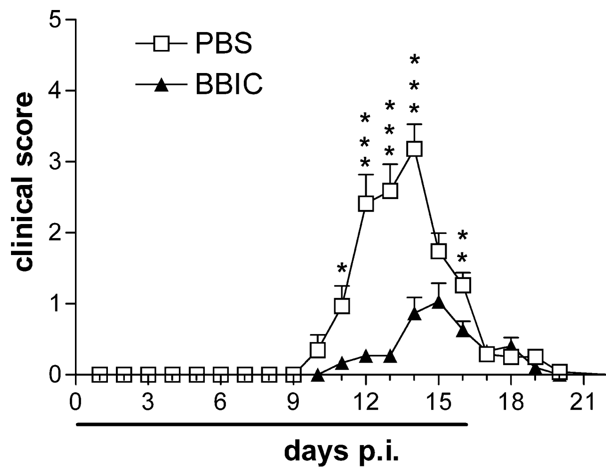

Figure 1 Effect of BBIC treatment from the day of immunization. Female Lewis rats were immunized with 100 μg GP-MBP in CFA. Rats received BBIC (200 mg/day, dissolved in 4 mL PBS, administered by gastric gavage; *n* = 15) from day 0 to 16 p.i. (the horizontal bar represents the duration of treatment) or PBS only (*n* = 17). Rats were scored daily for EAE severity by a blind observer. Data represent mean clinical scores ± SE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. One representative experiment of three is shown.

of disease, from day 8, 10, or 12 p.i. until sacrifice at day 20 p.i. In preliminary experiments, we found that BBIC at 200 mg/day did not significantly suppress EAE at the above mentioned time points (data not shown). We increased the dose to 400 mg BBIC/day (200 mg administered by gastric gavage twice daily). BBIC was most effective in suppressing EAE when administered from day 8 p.i. In this treatment group, disease onset was significantly delayed in comparison with PBS-treated rats ($P < 0.01$; Table 1). In addition, disease duration ($P < 0.001$), maximal severity ($P < 0.001$), and EAE-related weight loss ($P < 0.05$, data not shown) were also lower than in PBS-treated rats. Later treatment protocols significantly reduced maximal clinical scores. In addition, treatment started at day 10 p.i. significantly reduced disease duration, cumulative scores, and disease-related weight loss (Table 1 and data not shown).

Essential role of the protease-inhibitory activity of BBIC for clinical efficacy

To determine whether BBIC protease-inhibitory activity correlated with the therapeutic effect, we prepared HT-BBIC by autoclaving the drug solution twice before administration to rats. Thermal treatment has been shown to decrease the protease-inhibitory activity of BBIC [23]. Rats were immunized with MBP and divided into three treatment groups, which received PBS, BBIC, or HT-BBIC from the day of immunization until sacrifice on day 17 p.i. While BBIC significantly suppressed clinical EAE, HT-BBIC was not effective in reducing EAE severity (Table 2). These data indicate that the protease-inhibitory activity of BBIC is essential for its therapeutic efficacy in EAE.

BBIC reduces CNS inflammation

MBP-immunized rats were treated with BBIC (200 mg/day) or PBS from the day of immunization until sacrifice on day 16 p.i. Brains and spinal cords were harvested from rats after intracardiac perfusion and prepared for histological analysis of inflammatory infiltration. The numbers of mononuclear cell inflammatory infiltrates, as determined by HE staining (see Materials and methods), were significantly lower in brain and spinal cord sections in BBIC-treated than in PBS-treated rats (Figure 2A). Representative spinal cord and cerebellum sections from PBS- and BBIC-treated rats are shown in Figure 2B.

Effect of BBIC on antigen- and mitogen-induced T cell proliferation *in vitro* and *ex vivo*

To test whether BBIC inhibits the activation of autoreactive T cells induced by immunization with MBP, we sacrificed rats 15 days p.i. Draining lymph node cells were stimulated *in vitro* with MBP (1 μg/mL) or ConA (0.5 or 2.5 μg/mL) in the presence or absence of increasing doses of BBIC. BBIC inhibited MBP-induced proliferation of lymph node cells in a

Table 1 Effects of oral BBIC treatment (2 × 200 mg/day) from day 8–20, 10–20, and 12–20 p.i

Group (treatment)	Incidence[a] (%)	Day of onset (p.i., mean ± SD)	Disease duration (days, mean ± SD)	Max clinical score (mean ± SD)	Cumulative score (mean ± SD)
PBS	6/6 (100.0)	12.3 ± 0.8	5.8 ± 2.1	4.1 ± 0.2	15.9 ± 3.1
BBIC day 8–20 p.i.	5/6 (83.3)	13.8 ± 0.4**	2.2 ± 0.8***	1.8 ± 0.9***	3.5 ± 2.1***
BBIC day 10–20 p.i.	8/8 (100.0)	12.8 ± 0.5	3.5 ± 0.5*	2.9 ± 0.5***	8.3 ± 2.3**
BBIC day 12–20 p.i.	8/8 (100.0)	12.4 ± 1.1	4.1 ± 1.4	3.2 ± 0.7*	10.6 ± 5.2

[a]Disease incidence, number of rats that reached a clinical score of ≥ 1/total number of rats.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Mann–Whitney *U*-test).

Table 2 Effects of oral BBIC or heat-treated BBIC treatment (200 mg/day) from day 0–17

Group (treatment)	Incidence[a] (%)	Day of onset p.i. (mean ± SD)	Disease duration days (mean ± SD)	Max clinical score (mean ± SD)	Cumulative score (mean ± SD)
PBS	12/15 (80.0)	13.2 ± 1.5	4.9 ± 1.4	2.6 ± 1.6	8.8 ± 6.3
BBIC	16/36 (44.4)[b]	14.1 ± 0.5*	3.3 ± 0.9**	1.4 ± 1.4**	3.4 ± 3.7***
Heat-treated BBIC	10/10 (100.0)	14.0 ± 1.7	4.0 ± 1.6	2.8 ± 1.5	8.5 ± 5.8

[a]Disease incidence, number of rats that reached a clinical score of ≥1/total number of rats.
[b]$P = 0.03$ (Fisher's exact test).
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Mann–Whitney *U*-test).
Pooled data from two independent experiments.

dose-dependent manner (Figure 3). HT-BBIC significantly increased background proliferation, but did not inhibit MBP-induced proliferation. BBIC inhibited lymphocyte proliferation induced by a low dose of the T-cell mitogen ConA (0.5 μg/mL) in lymph node cells. Proliferation induced by a higher dose of ConA (2.5 μg/mL) was not affected (Figure 3).

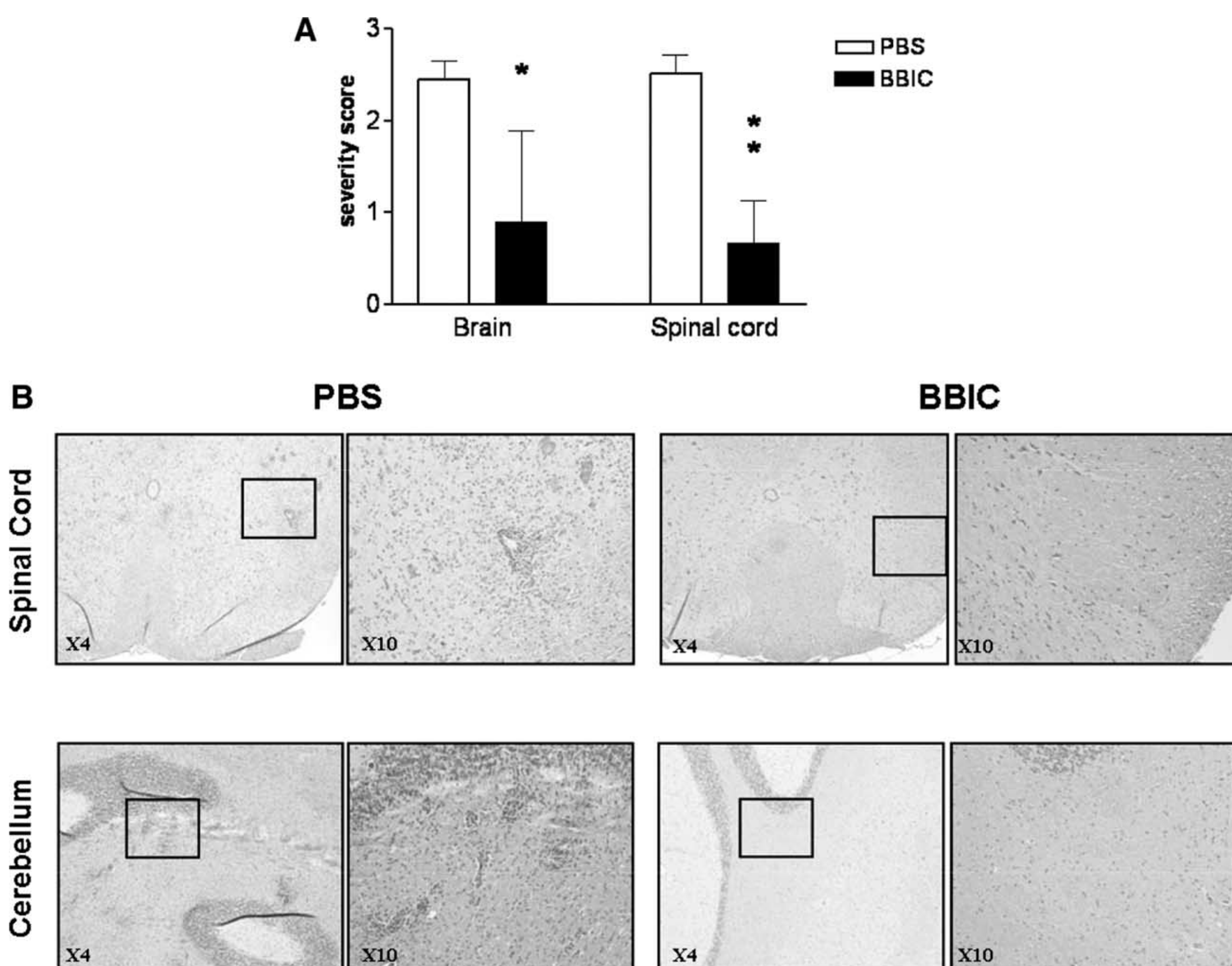

Figure 2 Histological examination of the central nervous system in PBS- and BBIC-treated rats. Rats were immunized for induction of active EAE and treated as shown in Figure 1 (200 mg BBIC or PBS from day 0 to 16 p.i.), sacrificed at day 16 p.i., and perfused by intracardiac injection of PBS. Histological evaluation of mononuclear cell inflammatory infiltrates was performed as described in Materials and methods after HE staining. (A) Data represent average severity scores ± SD in brain and spinal cord sections (*$P < 0.05$; **$P < 0.02$). (B) Representative microphotographs show inflammatory infiltration in spinal cord (upper panels) and cerebellum sections (lower panels) in PBS-(left-hand panels) and BBIC (right handed panel) treated rats. Magnifications are 4 × (first and third columns) and 10 × (second and fourth columns, areas indicated in boxes).

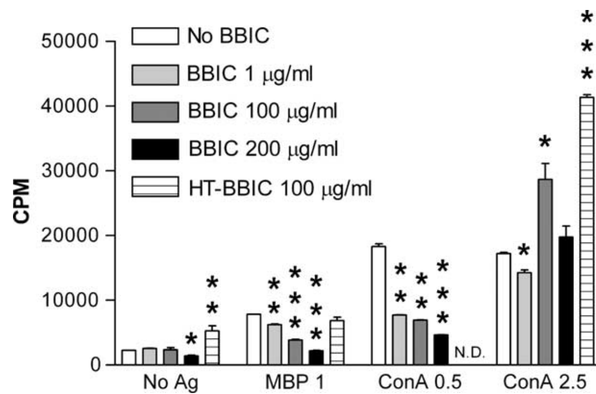

Figure 3 Effect of BBIC on antigen-induced T cell proliferation *in vitro*. Lymph node cells were isolated and pooled from MBP-immunized Lewis rats ($n = 4$, sacrificed at day 16 p.i.) and cultured (10^6 cells/well in 96-well plates) in the presence or absence of MBP (1 μg/mL) or ConA (0.5 or 2.5 μg/mL) and increasing concentrations of BBIC. Data represent mean values ± SD of one representative experiment out of two. *P* values refer to comparison between cell proliferation in the absence of BBIC and in the presence of different concentrations of BBIC. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

In *ex-vivo* experiments, we harvested lymph node cells from immunized rats treated with BBIC or PBS and sacrificed at day 10 or 14 p.i. and tested them for MBP- and ConA-induced proliferation without adding BBIC *in vitro* (Figure 4). Lymph node cell proliferation in the absence of antigen and in response to MBP was lower in rats treated with BBIC (200 mg/day) from day 0 to 10 p.i. compared to PBS-treated rats (Figure 4A). Proliferation in response to ConA did not differ between the two groups. Similar inhibition of proliferation was observed in lymph node cells isolated from rats treated with a higher dose of BBIC (2 × 200 mg/day) during the effector phase of disease (from day 8 p.i. until sacrifice at day 10 p.i.; Figure 4B). Finally, lymph node cells from rats sacrificed at day 14 p.i. showed decreased proliferation in response to MBP when rats were treated with BBIC starting from day 8 or 10 p.i (Figure 4C).

BBIC reduces the gelatinase activity of Lewis rat spleen cell supernatants

Due to the involvement of gelatinases in the induction of BBB damage in EAE and MS, we used zymography to test the effect of BBIC on gelatinase activity of spleen cell supernatants. We cultured spleen cells obtained from normal Lewis rats in serum-free RPMI medium in the presence or absence of 100 or 200 μg/mL BBIC for 72 hours. Supernatants cultured in the absence of BBIC produced bands of clearance co-migrating with recombinant MMP-2 (64 kDa) [27] and MMP-9 (84 kDa) [28] standards. Supernatants cultured in the presence of BBIC showed reduced MMP-2 and MMP-9 activity (Figure 5).

BBIC is detected in the CNS of EAE rats by Western blot analysis

To determine whether BBIC enters the CNS during EAE, we analysed brain homogenates of EAE and non-immunized control rats injected with BBIC or PBS by Western blot analysis. As a positive control, we used a BBI-specific antibody, 5G2, to detect major bands from a 10 mg BBIC/mL PBS solution. In addition to an 8 kDa band, corresponding to BBI monomers, we detected several other bands as previously reported (Figure 6A) [26]. EAE rats and healthy controls were then injected intracardially with 0.1 mg BBIC (dissolved in 1 mL PBS) or PBS only, sacrificed after 45 minutes and extensively perfused. Western blot analysis of brain homogenates identified BBI-specific 22 and 36 kDa bands in the CNS of EAE rats. Less intense bands were detected in the CNS of non-immunized control rats and no bands were detected in the CNS of non-immunized control rats injected with PBS (Figure 6B).

Discussion

In the present study, we tested the efficacy of the soy-derived serine protease inhibitor, BBI, in the treatment of EAE, an animal model for MS. Daily oral administration of BBIC – a soybean extract enriched in BBI [20] – from the day of immunization until sacrifice, consistently suppressed EAE, both clinically and histopathologically. In addition, treatment was also effective when started just before disease onset. These results suggest that BBIC may be a promising agent for oral therapy in MS patients. Oral BBIC was well tolerated in these experiments without detectable side effects.

BBIC inhibited the activation of T cells reactive to MBP *in vitro*. This may be due to inhibition of MHC class II antigen processing pathways. While cysteine and aspartic acid proteases can block intracellular processing pathways [29], serine protease inhibitors have been shown to block the generation of extracellular immunogenic peptides during inflammation [30]. Such activity may reduce the production and presentation of antigens that sustain chronic autoimmunity in EAE, MS, and other autoimmune diseases [7,9,31]. In addition to *in vitro* effects, our data also showed inhibition of MBP-specific T-cell activation *ex vivo*. Lymph node cell proliferation in response to MBP was significantly reduced, even when BBIC was administered for only three days before sacrifice (Figure 4B). Together, these data indicate that inhibition of

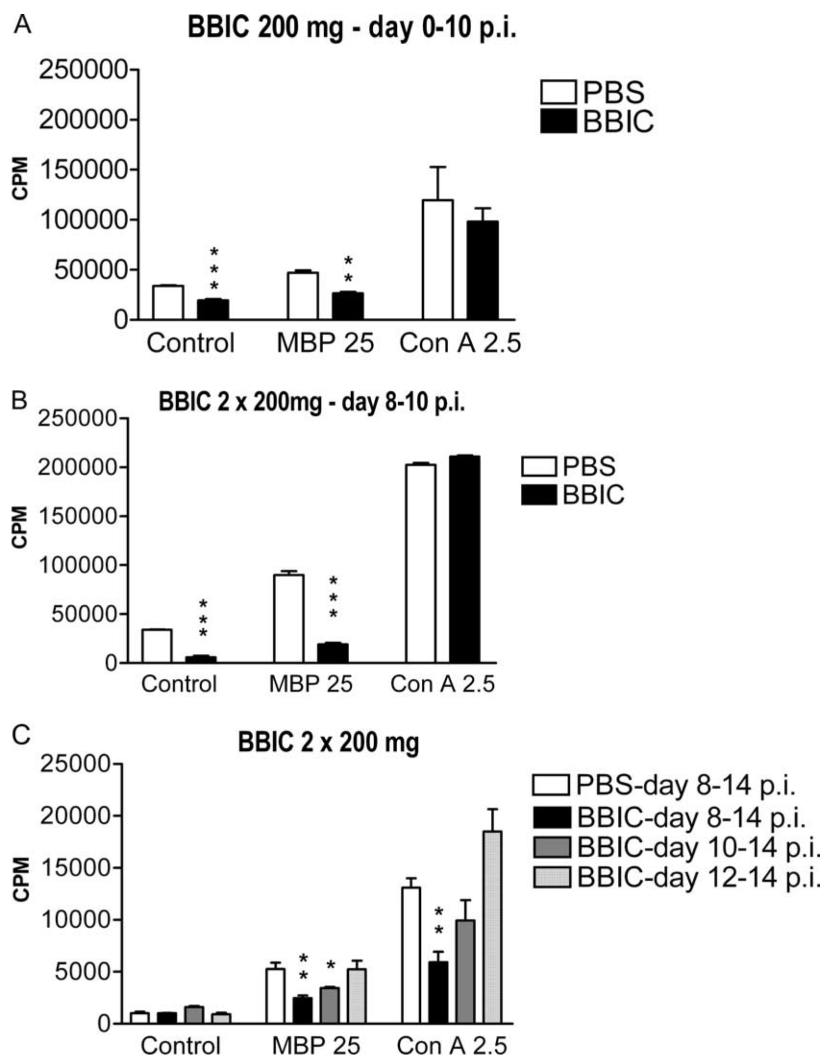

Figure 4 Effect of BBIC on antigen-induced T cell proliferation *ex vivo*. Lymph node cells were isolated and pooled from MBP-immunized Lewis rats treated with BBIC (200 or 400 mg/day) or PBS from the day of immunization (A) or from day 8 p.i. (B) and sacrificed at day 10 p.i. Lymph node cells (10tgfcol @|Set Fill Colour^6/well) were cultured in the presence or absence of MBP (25 μg/mL) or ConA (2.5 μg/mL) and then tested for antigen-induced proliferation without adding BBIC *in vitro*. In a separate experiment (C), lymph node cells were obtained from immunized rats treated with BBIC (400 mg/day) or PBS from day 8, 10, or 12 and sacrificed at day 14 p.i. Lymph node cells (2.5×10^5 cells/well) were cultured in the presence or absence of MBP (25 μg/mL) or ConA (2.5 μg/mL) and then tested for antigen-induced proliferation without adding BBIC *in vitro*. Data represent mean values ± SD of one representative experiment out of two. *P* values refer to comparisons between cell proliferation in rats treated with BBIC and PBS; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

T-cell activation by BBIC *in vivo* plays an important role in EAE suppression.

Two of our observations suggest that BBIC critically targets the effector phase of disease. First, although treatment including the first 7 days p.i. was more effective, it was not required for disease suppression. Second, there was a 'window' of therapeutic efficacy when treatment was started between day 8 and 10 p.i., after which efficacy was reduced (Table 1). Thus, the induction and the early effector phase of disease, which includes the entry of activated immune cells into the CNS, appear particularly sensitive to BBIC treatment.

Importantly, inhibition of clinical disease and T-cell activation *in vitro* required intact protease-inhibitory activity of BBIC, as indicated by heat-inactivation experiments (Table 2 and Figure 3) [23]. These data are supported by similar observations reported for the anti-carcinogenic activity of BBI [20].

Another target of BBIC action may be the permeability of the BBB. MMPs and other proteolytic enzymes are involved in increasing BBB permeability during inflammation through proteolysis of the basement membrane [32]. Since BBIC reduced MMP-2 and -9 activity in supernatants of

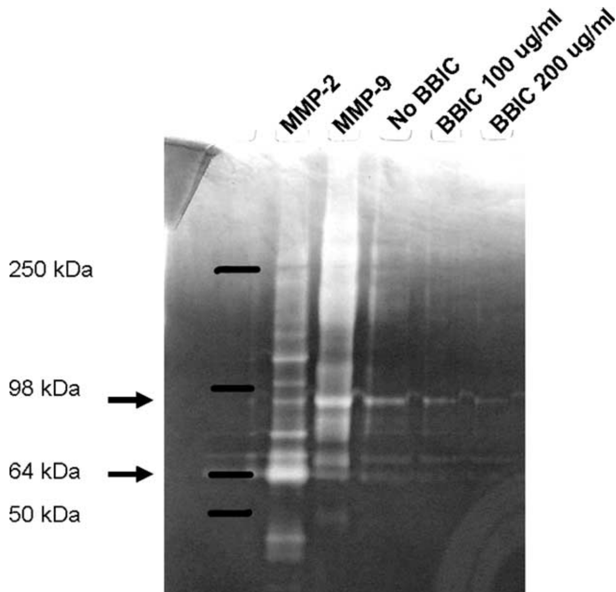

Figure 5 Effect of BBIC on gelatinase activity of spleen cell supernatants. Gelatinase activity was determined by zymographic analysis from samples of spleen cell supernatants cultured for 72 h in the presence or absence of 100 or 200 μg/mL BBIC, as described in Materials and methods. Clearance bands at 64 and 84 kDa (arrows) correspond to active MMP-2 and -9, respectively. The results are representative of two experiments.

spleen cells, it may contribute to limit MMP-induced BBB breakdown in EAE [33]. Consistent with our findings, BBI has been reported to inhibit the generation of active MMP-1 and -9 from their precursor pro-enzymes *in vitro* [34,35]. In addition, MMP-9 may be blocked by BBIC *in vivo*, as it has been shown to increase in the CSF of Lewis rats with EAE 9 days p.i. [14].

Orally administered BBIC survives the digestive process and is distributed throughout the body in the bioactive form [36,37]. Only traces of [^{125}I]-BBI were detected in the CNS of healthy CD-1 mice with intact BBB [36]. In our study, detection of BBI in brain homogenates of BBIC-injected rats indicates that the drug crosses the BBB in both healthy and EAE animals. A greater amount of BBIC was found in rats with EAE. The BBI/BBIC-specific antibody 5G2 detected bands in the range of 22 and 36 kDa. This suggests the presence of complexes of BBI bound to itself and to other components of BBIC, as previously reported [26], and possibly to CNS proteins. Together, these data suggest that (a) BBB damage may facilitate BBIC entry into the CNS during the effector phase of disease and modulation of inflammatory proteases in the target tissue [7]; and (b) BBIC would likely have free access to the CNS in MS patients. In the inflamed CNS, BBIC may reduce the generation of immuno-

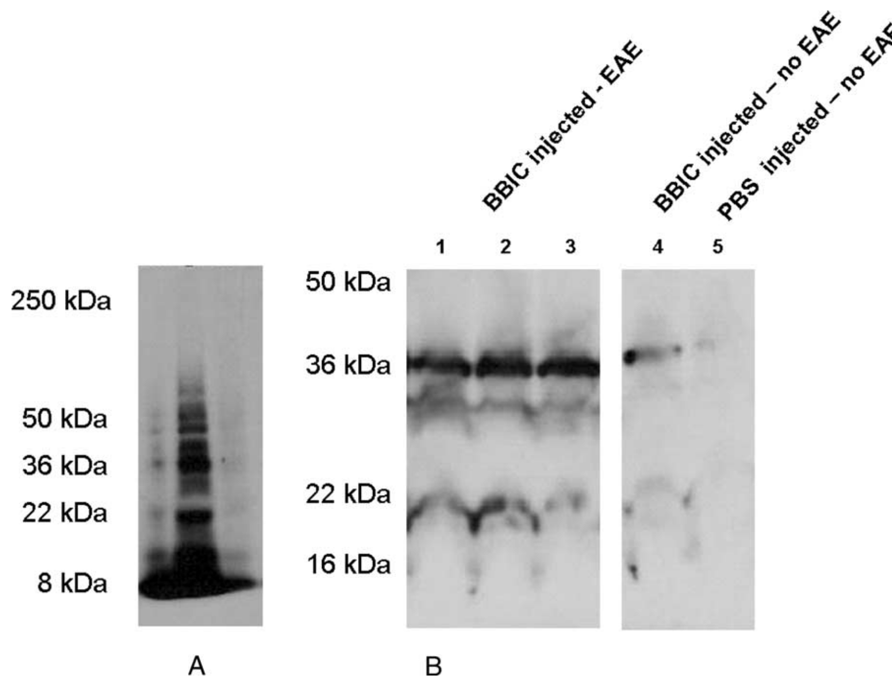

Figure 6 Detection of BBIC in brain homogenates by Western blot analysis. Western blot of a 10 mg BBIC/mL PBS solution was hybridized with 5G2 anti-BBI antibody. In addition to an 8 kDa band, corresponding to BBI monomers, other major bands are detected at 22 and 36 kDa (A). Western blot analysis of brain homogenates of rats injected intracardially with BBIC or PBS, sacrificed after 45 minutes, and extensively perfused, identifies BBIC-specific 22 and 36 kDa bands in the CNS (B). Lanes 1–3 illustrate EAE rats injected with BBIC. Lane 4 illustrates a non-EAE rat injected with BBIC. Lane 5 illustrates a non-EAE rat injected with PBS. One of two experiments with similar results is shown.

genic myelin peptides by leukocyte-derived elastase and cathepsin G [38], extracellular processing and presentation of antigens [9,30], as well as the production of reactive oxygen species [39], and mast cell-derived chymase [40,41].

Oral BBIC was very well tolerated in this study. This is an important and promising aspect of these findings with respect to potential use in patients. BBIC has shown a very favorable safety profile in pre-clinical studies [42,43], and in clinical trials [17]. No evidence of laboratory or clinical side effects was observed in phase I/II trials of BBIC in patients with oral leukoplakia, benign prostatic hyperplasia, and in a phase II study in patients with ulcerative colitis, an immune-mediated form of inflammatory bowel disease (IBD) [44–46]. The serum half-life of 10 hours after oral administration in experimental animals [18], suggests that one daily oral dose of BBIC used in previous clinical trials would be appropriate in MS patients [17].

Frequent drug injections are sometimes a problem for MS patients, and it would be advantageous to add an oral agent to the repertoire of MS drugs. This could be used as a monotherapy or as an addition to disease-modifying drugs [47]. Importantly, BBIC has attained investigational new drug (IND) status with the FDA, and trials to evaluate it as an anti-carcinogenic agent in human populations have been ongoing for over a decade with encouraging clinical efficacy and minimal toxicity [17,44–46].

We observed similar efficacy of BBIC in the closely related experimental autoimmune neuritis (EAN), a CD4+ T cell mediated animal model of the Guillain–Barre' syndrome (GBS) in human patients (Rostami *et al*., manuscript in preparation), indicating that BBIC warrants further investigation in the field of inflammatory demyelinating peripheral neuropathy as well.

We conclude that oral BBIC, an effective, well tolerated, and relatively inexpensive treatment for EAE, should be further tested for the evaluation of safety in a phase I/II clinical trial in MS patients.

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