Antigen-specific Suppression of Experimental Autoimmune Encephalomyelitis by a Novel Bifunctional Peptide Inhibitor: Structure Optimization and Pharmacokinetics

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Abbreviations: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; PLP, proteolipid protein; BPI, bifunctional peptide inhibitor; LFA-1, lymphocyte function-associated antigen-1; ICAM-1, intercellular adhesion molecule-1; MHC-II, major histocompatibility complex-II; APC, antigen presenting cells; TCR, T cell receptor; IL-17, interleukin-17; AUC, area under curve; C_{max}, maximum plasma concentration; Cl, systemic clearance; t_{1/2}, half-life
Abstract

The objective of this study was to optimize the *in vivo* activity of PLP-BPI molecule to suppress EAE in SJL/J mice and evaluate pharmacokinetic profiles of PLP-BPI. PLP-BPI is constructed *via* conjugation of myelin proteolipid protein (PLP\textsubscript{139-151}) with CD11\textalpha\textsubscript{237-246}-derived peptide (LABL) *via* a spacer. The hypothesis is that PLP-BPI binds simultaneously to MHC-II and ICAM-1 on the antigen-presenting cell and inhibits the formation of the immunological synapse during T cell and antigen-presenting cell interactions. In this study, the structure of BPI was modified by varying the spacer and was evaluated in the EAE model. Intravenous injections of BPI derivatives inhibited the onset, severity, and incidence of EAE more effectively as well as inducing a lower incidence of anaphylaxis than produced by unmodified PLP-BPI. As anticipated, production of IL-17, a proinflammatory cytokine commonly found in elevated levels among MS patients, was significantly lower in Ac-PLP-BPI-PEG6- or Ac-PLP-BPI-NH\textsubscript{2}-2-treated mice than in PBS-treated mice. These results suggest that BPI-type molecules can be modified to achieve more efficient and better tolerated BPI-based derivatives for the treatment of MS.
Multiple sclerosis (MS), a demyelinating disease affecting the CNS, is characterized by inflammatory demyelinating foci in the brain white matter with variable axonal damage (Huizinga et al., 2008; Rueda et al., 2008). Although its etiology is still scarcely understood, the composition of plaques, immunogenetic background, responses to immunomodulation, and data from animal models suggest that MS is mediated by myelin-specific CD4+ T cells (Lu, 2006; Sonobe et al., 2007; Elyaman et al., 2008; Sabatino et al., 2008). It is believed that in autoimmune diseases (e.g., MS, type-1-diabetes, and rheumatoid arthritis), the activation of autoreactive T cells is due to the recognition of the self antigen (i.e., fragments of self-protein) prior to the attack of self organs. In the case of MS, the activation of a subpopulation of T cells may cause damage to the central nervous systems by attacking the myelin sheath proteins on the nerve fibers.

Currently, MS patients are being treated with anti-inflammatory agents (e.g., corticosteroids, interferon, mitoxantrone), Copaxone®, and Tysabri®. Copaxone® is a synthetic peptide polymer designed as a decoy for the myelin basic protein (MBP); it has been suggested to alter the immune response from Th1 type to Th2 type (Johnson, 1996). TYSABRI® is a recombinant human monoclonal antibody (mAb) that inhibits leukocyte adhesion by binding to the α4-subunit of α4β1 and α4β7 integrins on the surface of leukocytes except neutrophils (Langer-Gould et al., 2005; Kappos et al., 2007). Due to fatalities in two patients who were infected with JC virus in the brain, TYSABRI® was once withdrawn from the market but later reinstated for clinical use with some cautions. It was speculated that blocking the leukocyte adhesion signal (Signal-2) may have compromised the general immune response, including the subpopulation that can fight viral infections. Thus, there is an urgent need to develop therapies that can selectively suppress a
subpopulation of T cells that causes the progression of the autoimmune diseases without compromising the general immune response.

The initiating event in the activation of autoreactive CD4+ T cells is triggered by the interaction between T cells and antigen-presenting cells (APC) through receptor/ligand-like molecular combinations widely referred to as Signal-1 and Signal-2 (Grakoui et al., 1999; Bromley et al., 2001). Signal-1 includes the interaction between T-cell receptors (TCR) on the surface of T cells and antigen-loaded major histocompatibility complex (MHC) on the surface of APC (Frauwirth and Thompson, 2002). Signal-2, also referred to as the costimulatory signal, can be due to interaction of a variety of molecular pairs such as lymphocyte function-associated antigen (LFA)-1/intercellular adhesion molecule (ICAM)-1, CD28/B7, CTLA-4/B7, and inducible costimulatory/inducible costimulator ligands, and PD-1/PD-1 ligands (Grakoui et al., 1999; Schaub et al., 1999; Bromley and Dustin, 2002). Initially, the TCR: MHC-peptide complexes (Signal-1) are formed in the peripheral region, and the ICAM-1/LFA-1 complexes (Signal-2) are formed in the central region. Then, the ICAM-1/LFA-1 clusters translocate to the outer region and the TCR: MHC-peptide complexes congregate at the center to form the inner region. In the final state, TCR:MHC-peptide complexes congregate at the center to form the central supramolecular activation clusters (cSMAC) and the LFA-1/ICAM-1 complexes form a ring around the inner region to form peripheral supramolecular clusters (pSMAC). A combination of cSMAC (Signal-1) and pSMAC (Signal-2) is called the immunological synapse (Monks et al., 1998; Grakoui et al., 1999; Lee et al., 2002).

We discovered a novel and selective way to suppress autoimmune diseases (i.e., MS and type-1 diabetes) using a bifunctional peptide inhibitor (BPI), which consists of an antigen peptide
epitope and an ICAM-1-binding peptide conjugated via a spacer (Kobayashi et al., 2007, 2008; Murray et al., 2007). Although the mechanism of action of BPI is still unknown, we hypothesize that BPI molecules block the formation of the immunological synapse by simultaneously binding to the MHC-II and ICAM-1 on the APC to prevent the translocation and segregation of Signal-1 and Signal-2 during the formation of the immunological synapse (Kobayashi et al., 2007, 2008; Murray et al., 2007). In this case, the antigenic-peptide epitope 139–151 derived from the proteolipid protein (PLP139–151) was conjugated to LABL peptide derived from αL integrin (CD11a237–246) to make BPI molecules (Kobayashi et al., 2007, 2008). To optimize the efficacy and lower the potential side effects of PLP-BPI molecules, the linker between the PLP and LABL peptides was modified and the pharmacokinetic properties of the BPI molecule were evaluated. The effect of linker modification of BPI on the prophylactic and therapeutic activities was determined. The data of the pharmacokinetic profiles will be used to assess the safety profile of the BPI molecule and to design future formulation and delivery methods for this type of molecule.
Materials and methods

**Peptide synthesis.** Peptides used in the present study are listed in Table 1. The peptides were synthesized with 9-fluorenyl-methoxy-carbonyl-protected amino acid chemistry on appropriate PEG-PS™ resin (GenScript Corporation, Piscataway, NJ) using an automated peptide synthesizer (Pioneer; Perceptive Biosystems, Framingham, MA). Cleavage of the peptides from the resin and removal of the protecting groups from the side-chain were carried out using trifluoroacetic acid with scavengers. The crude peptides were purified by reversed-phase high performance liquid chromatography (HPLC) using a preparatory C_{18} column with a gradient of solvent A [95%/5%, H_{2}O (0.1% trifluoroacetic acid)/acetonitrile] and solvent B (100% acetonitrile). The purity of the peptides was analyzed by HPLC using an analytical C_{18} column. The identity of the synthesized peptide was confirmed by matrix-assisted laser desorption ionization/time of flight mass spectrometry.

**Mice.** SJL/J (H-2^S) female mice were purchased from Charles River (Wilmington, MA) and housed under specific pathogen-free conditions at the Association for Assessment and Accreditation of Laboratory Animal Care-approved facility at the University of Kansas. All protocols involving live mice were approved by the University’s Institutional Animal Care and Use Committee.

**Induction of EAE and therapeutic study.** Five-to-seven-week-old SJL/J female mice were immunized s.c. with 200 µg of PLP_{139-151} in a 0.2-ml emulsion composed of equal volumes of phosphate-buffered saline (PBS) and complete Freund’s adjuvant (CFA) containing killed *Mycobacterium tuberculosis* strain H37RA (at a final concentration of 4 mg/ml; Difco, Detroit,
MI). The PLP_{139-151}/CFA was administered to regions above the shoulder and the flanks (total of four sites; 50 µl at each injection site). In addition, 200 ng of pertussis toxin (List Biological Laboratories Inc., Campbell, CA) was injected i.p. on the day of immunization (day 0) and 2 days post-immunization. The mice received i.v. injections of peptides (100 nmol/mouse) on indicated day(s). For the therapeutic study, mice were left untreated until the day of disease onset (determined when the mouse had an EAE clinical score of one or more for the first time). Upon disease onset, the mice received i.v. injections of peptides (100 nmol/mouse) for 3 consecutive days of maximum number of injections; peptide injection was discontinued once the disease score returned below 1. Disease progression was evaluated by the same observer in a blinded fashion using a clinical scoring as follows: 0, no clinical signs of the disease; 1, tail weakness or limp tail; 2, paraparesis (weakness or partial paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund (mice were euthanized once they were found to be moribund). Body weight was also measured daily.

**Determination of IL-17 Levels in Serum in vivo.** Blood samples were obtained from peptide-treated and untreated mice on day 12 and day 35 (6 mice per group). Additionally, 6 unprimed and untreated mice (i.e., normal mice) were also sampled. Blood samples were allowed to clot overnight at 2–8°C before centrifuging for 20 min at 2000 × g. Serum was collected and stored at −20°C until analysis. Enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, MN) for IL-17 in serum were performed according to the manufacturer’s instructions. Optical density was measured at 450 nm with correction at 540 nm

**Induction and Monitoring of Anaphylaxis.** Mice received s.c. immunization with PLP_{139-151}/CFA on day 0, and i.p. injection of pertussis toxin on the day of immunization and 2 days post-immunization. Four to five weeks later, the mice were divided into groups and received intravenous injection of described peptides (100 nmol/mouse). To avoid the influence of the variation in their disease severity and history, all the groups had a very similar set of mice in terms of the average highest disease score, the average cumulative disease score, and the average day of disease onset. Incidence of anaphylactic response was judged by death occurring within 30 min or by the characteristic signs of immediate hypersensitivity, such as piloerection; prostration; erythema of the tail, ears, and footpads; shallow breathing; and lethargy, observed within a few minutes after peptide injection. Any mice that became moribund or did not recover from anaphylactic signs were euthanized.

**In vivo Pharmacokinetic Studies in Rats.** Male Sprague-Dawley rats weighing 250–300 g were purchased from Charles River (Wilmington, MA) and housed under specific pathogen-free conditions at the Association for Assessment and Accreditation of Laboratory Animal Care-approved facility at the University of Kansas. All protocols involving live rats were approved by University’s Institutional Animal Care and Use Committee. Prior to surgery, the animals were anesthetized with intraperitoneally administered ketamine (100 mg/kg) and xylazine (5 mg/kg). The right jugular vein was exposed and cannulated with medical-grade silastic tubing (0.020 in., id × 0.037 in., od). The vein was ligated with a 2–0 silk suture and anchored to the surrounding tissue with cyanoacrylate glue. The patency of the jugular catheter was maintained with sterile
saline containing heparin (10 U/ml). The animal was maintained unconscious throughout the experiment by subcutaneously administered 25 mg/kg of ketamine. Baseline “blank” plasma was drawn from each animal immediately before dosing. An intravenous bolus dose was administered via the jugular vein over a period of 30 s. After dosing, the jugular vein was flushed with sterile saline. Blood samples (~0.2 mL) were drawn from the jugular vein at scheduled time intervals for 10 h after dosing. The blood samples were immediately centrifuged at 10,000 g for 3 min, plasma separated, and stored at –70°C until analyzed by LC-MS/MS.

**Plasma Extraction Procedure.** To 95 µl of drug free rat plasma, 5 µl of working solutions of Ac-PLP-BPI-NH₂-2 and Ac-LABL-BPI-NH₂ (internal standard) were spiked to yield final concentrations in the range of 15–1000 pmol. Quality control (QC) samples were prepared in a similar manner. All samples, QCs, and standards were diluted with Nanopure water to 1 mL, vortexed for 30 s, and isolated from plasma by solid-phase extraction (Oasis HLB®, Waters Corp., Milford, MA). Solid-phase extraction cartridges were pretreated with 1 mL of methanol followed by 1 mL of water before the diluted samples were loaded. After running the samples through the cartridges, the cartridges were washed twice with 1.0 mL of 10% acetonitrile in water. Samples were eluted with 1.0 mL of 60% methanol with 2% TFA in water, evaporated under nitrogen, and reconstituted with 100 µL of water: acetonitrile (1:1, v/v).

**LC-MS/MS Conditions.** The quantity of peptides recovered from plasma was determined by LC/MS/MS using a small, short C₁₈ guard column and monitoring an abundant product ion from activation of a multiply charge precursor of the peptide. A Waters Acquity UPLC (Waters, Milford, MA) interfaced with Micromass “triple” quadrupole (Quattro Ultima Micromass Ltd. Manchester UK) mass spectrometer was used. Samples were presented using a Zorbax C₁₈ (0.32
mm × 25 mm, 5 µm) column (Micro-Tech Scientific, Sunnyvale, CA) heated to 35°C and eluted with a mobile phase gradient at a flow rate of 60 µl/min. The Injection volume was 10 µL. Mobile phase consisted of A (H2O with 1% CH3CN) and B (CH3CN with 0.08% formic acid). The gradient was 10% mobile phase B isocratic for 2 min, increased to 60% B in 1 min then held at 60% B for 3 min. Multiple reaction monitoring (MRM) mode was used for the quantification. The electrospray source block was 80 °C and probe desolvation temperature 200 °C. Argon collision gas was set to attenuate the beam to 15% (8e-4 mbar on a gauge near the collision cell). The cone voltage was 25 V. Quadrupoles 1 and 3 were tuned to a resolution of 0.8 u FWHH. The selected transitions were from the M4H4 precursors, m/z (855.1>724) collision energy 28 V for Ac-PLP-BPI-NH2-2 and m/z 988.5>1059.8 collision energy 35V for Ac-LABL-BPI-NH2 (internal standard). Because of the high charge state of the precursor the optimum collision gas density and energy occur over a narrower range than commonly observed for singly charged ions.

Pharmacokinetic Data Analysis. Pharmacokinetic analysis of Ac-PLP-BPI-NH2-2 plasma concentration-time profiles after IV bolus injection was conducted by fitting the data to a two-compartment pharmacokinetic model using WinNonlin™ Professional Version 3.1 (Pharsight Corporation, Mountain View, CA) according to Eq. 1 below:

\[ C = Ae^{-\alpha t} + Be^{-\beta t} \]  

\[ (1) \]

Similarly, the initial volume of distribution (V_initial) was calculated according to Eq. 2:

\[ V_{\text{initial}} = \frac{D}{C_{\text{max}}} \]  

\[ (2) \]
Where C is the plasma concentration of Ac-PLP-BPI-NH₂-2; A and B are preexponential constants; \( \alpha \) is the distribution rate constant; \( \beta \) is the elimination rate constant; \( t \) is time; \( D \) is the dose; and \( C_{\text{max}} \) is the maximum plasma concentration. The parameters that were calculated for Ac-PLP-BPI-NH₂-2 included \( C_{\text{max}} \), \( AUC_{0-\infty} \), Cl, and \( t_{1/2} \). Pharmacokinetic parameters were derived for each animal based on individual observed concentration-time data.

**Statistical Analysis.** Statistical comparisons among the groups in clinical disease scores were determined by calculating the average score for each mouse from the day of disease onset to the end of the study and performing a Mann-Whitney’s U test. Statistical differences among the groups in body weight were analyzed by calculating the average weight for each mouse for 10 days beginning on the day of disease onset and performing a Mann-Whitney’s U test. Statistical significance in EAE disease morbidity was determined by Cox proportional-hazards regression. Comparison of IL-17 concentration in serum was performed by one-way analysis of variance. All analyses were carried out using StatView (SAS Institute, Inc., Cary, NC).
Results

Effect of Linker Modification in BPI on the Suppression of EAE. The effects of the nature of the linker and its length were evaluated to optimize the in vivo efficacy of the BPI molecules. The hypothesis is that modification of linker properties will influence the efficacy and side effects of the BPI molecules. In this case, the Acp-G linker in Ac-PLP-BPI-NH$_2$-2 was modified with polyethylene glycol (PEG) with different lengths to give Ac-PLP-BPI-PEG3 and Ac-PLP-BPI-PEG6 (Table 1). The in vivo activities of Ac-PLP-BPI-PEG3 and Ac-PLP-BPI-PEG6, Ac-PLP-BPI-NH$_2$-2 were compared to PBS in therapeutic experiments using immunized SJL/J mice. In this case, the animals received i.v. injections of the peptides or PBS after they showed disease signs with a score of one or higher. The mice received i.v. injections of the peptides or PBS on consecutive days until the score was less than one or a maximum of three injections of the peptides, whichever came first. All three peptides reversed the diseases progression more effectively compared to the control (Fig. 1A) with the following statistical differences in EAE clinical scores on days 10–25: Ac-PLP-BPI-NH$_2$-2 vs. PBS with ***$p < 0.001$; Ac-PLP-BPI-PEG6 vs. PBS with ***$p < 0.001$; and Ac-PLP-BPI-PEG3 vs. PBS with ***$p < 0.001$. Ac-PLP-BPI-NH$_2$-2-treated mice recovered from EAE more rapidly compared to those treated with the other two peptides (Ac-PLP-BPI-NH$_2$-2 vs. Ac-PLP-BPI-PEG3, **$p < 0.01$; Ac-PLP-BPI-NH$_2$-2 vs. Ac-PLP-BPI-PEG6, *$p < 0.05$) as shown by the rapid decline in their clinical scores particularly from days 10-15 (Fig 1A). Additionally, Ac-PLP-BPI-NH$_2$-2-treated mice did not lose as much body weight and/or regained their body weight faster (***$p < 0.001$) than mice treated with Ac-PLP-BPI-PEG3 (Fig 1B). Although there was no statistical difference in loss of body weight between Ac-PLP-BPI-NH$_2$-2- and Ac-PLP-BPI-PEG6-treated
mice (*p > 0.05), there was a trend that Ac-PLP-BPI-NH$_2$-2-treated mice regained their body weight faster than Ac-PLP-BPI-PEG6-treated mice (Fig 1B). No statistical difference (*p > 0.05) was observed in EAE clinical scores data between Ac-PLP-BPI-PEG6 and Ac-PLP-BPI-PEG3. However, Ac-PLP-BPI-PEG3-treated mice had significantly (***p < 0.001) higher loss in body weight compared to Ac-PLP-BPI-PEG6-treated mice (Fig. 1B); suggesting that Ac-PLP-BPI-PEG3 did not work as well as Ac-PLP-BPI-PEG6. Thus, no further studies were conducted on Ac-PLP-BPI-PEG3 molecule.

The therapeutic experiments were further corroborated by prophylactic experiments. In this case, the peptides were administered via i.v. route on days 4 and 7 prior to the onset of EAE. Ac-PLP-BPI-NH$_2$-2-treated mice showed clinical scores of mild EAE compared to the severe EAE found in PBS-treated mice, particularly at the peak of the disease on days 12–17 (***p < 0.001) (Fig. 2A). Ac-PLP-BPI-NH$_2$-2 treated mice had significantly (***p < 0.001) minimal loss of body weight compared to the control as shown by Fig. 2B. At the peak of disease severity (days 12–17), PBS treated mice had as much as 24% loss in body weight compared to a maximum 11% loss in body weight among the Ac-PLP-BPI-NH$_2$-2 treated mice (Fig. 2B). Similarly, i.v. injections of Ac-PLP-BPI-PEG6 inhibited the progression and severity of EAE similar to that of Ac-PLP-BPI-NH$_2$-2 (*p > 0.05). The PBS-treated mice recorded EAE clinical scores as high as 4, which included paralysis of their tails and limbs. To examine the necessity of the correct peptide sequence of the BPI molecule, an Ac-PLP-BPI-PEG$_{SC}$ peptide with scrambled sequences at both PLP and LABL fragments was used as a control. This peptide did not suppress the progress of EAE (Fig 2A and Fig 2B); suggesting that the correct sequences of both peptides is necessary for the activity to suppress EAE.
The effect of linker modification on reducing the anaphylaxis side effect of the peptides was evaluated by multiple injections of the peptides on the treated animals in the late phase of the disease (i.e., 35 days) (Table 2). The animals treated with Ac-PLP-BPI-PEG6 have lower anaphylactic response (22%, Table 2) compared to those of treated with the Ac-PLP-BPI-NH2-2 (45%). As found previously, the animals treated with PLP_{139-151} had the highest anaphylactic response (87%). This suggests that BPI molecules have lower side effects than the plain PLP_{139-151}; in addition, changing the linker from Acp-G repeat to PEG repeat lowers the side effects of the BPI molecules. As a control, the scrambled peptide (i.e., Ac-PLP-BPI-PEG6SC) did not induce any anaphylactic reaction (0%). Studies by Kuchroo et al. identified specific residues in the antigenic core that are TCR binding sites and those that are MHC-II binding sites (Kuchroo et al., 1994). Their findings indicated that residues Trp-144 and His-147 were the TCR binding sites whereas residues Leu-145 and Pro-148 were important for MHC-II binding. Thus, any changes in the position of these amino acids will affect the MHC-II- and TCR-binding properties as well as peptide activity. Because the sequence in Ac-PLP-BPI-PEG_{SC} is scrambled, it is expected that Ac-PLP-BPI-PEG_{SC} has no suppressive activity and anaphylactic reaction in the EAE mouse model.

**IL-17 Serum Levels in SJL/J Mice In Vivo.** The effect of linker modification of BPI molecules on suppressing the IL-17 levels was evaluated because IL-17 is a marker for the progress of multiple sclerosis. In this study, the animals were treated with Ac-PLP-BPI-NH2-2, Ac-PLP-BPI-PEG6, or PBS on days 4, 7, and 10 and IL-17 levels measured on day 12 and 35 (Fig. 3A). As expected, the clinical scores, body weight changes, and disease incidence (Fig. 3)
indicated that both peptides have better efficacy than PBS. Three injections of the peptides provided lower disease incidence (Fig. 3D) than did 2 injections on days 4 and 7 (Fig. 2C). In the mice treated with Ac-PLP-BPI-NH₂-2 or Ac-PLP-BPI-PEG6, the amount of IL-17 was significantly decreased from day 12 to day 35; in contrast, the mice treated with PBS had higher levels of IL-17 on day 35 than on day 12. However on day 12, mice receiving Ac-PLP-BPI-NH₂-2 or Ac-PLP-BPI-PEG6 had unexpected significantly high serum levels of IL-17 compared to the mice receiving PBS alone (**p < 0.001) (Fig. 3A). At the end of the study (day 35), mice receiving Ac-PLP-BPI-NH₂-2 or Ac-PLP-BPI-PEG6 had significantly lower IL-17 serum concentration (**p < 0.01) than did PBS-treated mice. Although there was a trend that the level of IL-17 was lower in Ac-PLP-BPI-PEG6-treated group than in Ac-PLP-BPI-NH₂-2-treated group on day 35, there was no significant difference (*p > 0.05) between these levels of IL-17. Measured serum levels of IL-17 in the unprimed mice were found to be 2.77 ± 4.48 pg/mL (Fig. 3D), representing the background level of IL-17 in mouse. As anticipated, these levels were the lowest IL-17 serum concentration among all the groups.

**Pharmacokinetic Analysis of Ac-PLP-BPI-NH₂-2 in Sprague-Dawley Rats.** For pharmacokinetic studies, plasma concentrations of Ac-PLP-BPI-NH₂-2 were determined using LC-MS/MS. We chose Ac-PLP-BPI-NH₂-2 as our model BPI compound to study pharmacokinetics because we had done extensive studies on this compound in terms of its efficacy, potential to cause hypersensitivity, and cytokine profiling after treatment in our prior experiments. The initial step in analysis of extracted plasma samples involved the development and validation of the LC-MS/MS analytical method. It was found that a gradient mobile phase...
composition provided good resolution and symmetric peak shapes of analytes as well as short run time. The retention times were 2.44 and 3.48 min for Ac-PLP-BPI-NH$_2$-2 and internal standard (IS), respectively. No significant interfering peaks due to endogenous compounds were observed at the retention times of the two analytes of interest. The standard calibration curves of Ac-PLP-BPI-NH$_2$-2 in plasma were linear over the concentration range of 15–1000 pmol. Typical mean (n = 3) calibration curves were $y = 0.0066x - 0.6754$, $R^2 = 0.999$, where $y$ and $x$ are the peak area ratio of analyte to internal standard and concentration, respectively. The mean plasma extraction recoveries for Ac-PLP-BPI-NH$_2$-2 and Ac-LABL-BPI-NH$_2$ (IS) determined in triplicate in the concentration range of 15–1000 pmol were 91.8% (%CV 5.8) and 98.4% (%CV 4.3%), respectively. The peak areas of the analytes in the reconstituted QC samples had a coefficient of variation of less than 10%, indicating that the extracts were “clean” with no co-eluting compounds that could influence ionization of the analyte or the IS. The lower limit of quantification (LLOQ) defined as the concentration of Ac-PLP-BPI-NH$_2$-2 that can still be determined with acceptable precision (%CV <10) and accuracy (FDA et al., May 2001), was found to be 75 pmol, and the limit of detection (LOD) was 35 pmol. Results of the intra-day and inter-day validation assays indicated that the accuracy of the assay was >90% with a coefficient of variation that did not exceed 10%. For at least 48 h at the autosampler temperature (12°C), no significant degradation was observed in the extracted and reconstituted plasma samples as well as in working solutions. Ac-PLP-BPI-NH$_2$-2 and the IS in the final extract were considered to be stable when 85–115% of the initial concentration was found.

The validated LC-MS/MS method was applied to a pharmacokinetic study of Ac-PLP-BPI-NH$_2$-2 after administration of intravenous injections in Sprague-Dawley rats. Mean plasma
concentration-time profiles of Ac-PLP-BPI-NH₂-2 after i.v. injections of 0.3, 0.9, 1.8, and 5.0 µmol/kg are shown in Figure 4 and the related pharmacokinetic parameters are listed on Table 3. Observed plasma concentration-time profile followed a two-compartmental model characterized by a rapid distribution phase and a gradual elimination phase, and had a good correlation ($R^2 = 0.9872$) with the predicted plasma concentration. Maximum Ac-PLP-BPI-NH₂-2 plasma concentrations ($C_{\text{max}}$) of 3.84 ± 1.03, 18.17 ± 4.50, 69.59 ± 19.69, and 116.05 ± 24.92 nmol/mL were observed at 0.3, 0.9, 1.8, and 5.0 µmol/kg doses, respectively.
Discussion

In our recent studies, it was found that *in vivo* activity of PLP-BPI could be improved further by increasing its plasma stability as well as optimizing the separation distance between PLP and LABL peptides. Because the original PLP-BPI has uncapped N- and C-termini, it may be susceptible to amino- and carboxy-peptidase degradation in the blood. The rate of degradation can be decreased tremendously or halted by capping the N- and C-termini by acetylation and amidation, respectively. In our observations, a single injection of Ac-PLP-BPI-NH$_2$ with capped N- and C-termini inhibited the onset and progression of EAE more efficiently than uncapped PLP-BPI (Kobayashi et al., 2008), and there was no significant difference between Ac-PLP-BPI-NH$_2$ and PLP-BPI upon four injection of these two different peptides. It is plausible that the improved efficacy of Ac-PLP-BPI-NH$_2$ is due to increased bioavailability and lower metabolic degradation compared to the parent PLP-BPI. Ac-PLP-BPI-NH$_2$ was further optimized by doubling the linker length to give Ac-PLP-BPI-NH$_2$-2. *In vivo* studies in SJL/J mice revealed that Ac-PLP-BPI-NH$_2$-2 has activity similar to that of Ac-PLP-BPI-NH$_2$ but lower anaphylaxis reaction, suggesting that the linker properties and length may have a role in the hypersensitivity reaction of the BPI molecules.

The therapeutic effectiveness and the presence of potential side effects of our BPI molecules could also be due to their pharmacokinetic properties. Therefore, to move closer to clinical use of these peptides, we need to evaluate their pharmacokinetic profiles. For practical reasons such as the need for frequent sampling and sampling volume, male Spraque-Dawley rats were used instead of mice. In the present study, we conducted pharmacokinetic studies of Ac-PLP-BPI-NH$_2$-2 after intravenous administration. Mean plasma concentration-time profiles of Ac-PLP-
BPI-NH₂-2 after i.v., injections of 0.3, 0.9, 1.8, and 5.0 µmol/kg are shown Fig. 4, and the related pharmacokinetic parameters are listed in Table 3. Although there was no proportionality, there was an increase in the maximum plasma concentration (C_max) with an increase in the dose administered. This trend was also observed in the AUC values, suggesting that systemic exposure (C_max and AUC) of Ac-PLP-BPI-NH₂-2 increased with an increase in dose administered. Except for the smaller clearance values at the 0.3 µmol/kg dose, there were no significant differences (*p > 0.05) in the clearance values and half-lives (t₁/₂) at 0.3, 0.9, 1.8, and 5.0 µmol/kg doses. This fairly dose-independent disposition (Cl and t₁/₂) of Ac-PLP-BPI-NH₂-2 suggests non-saturable hepatic extraction. As with any peptide, Ac-PLP-BPI-NH₂-2 is expected to have extensive hepatic extraction and, in the case of saturable hepatic extraction, we anticipate that the clearance values should increase with a decrease in the dose administered. On the other hand, elimination half-lives (t₁/₂) are expected to decrease with a decrease in the dose administered. However, the observed pharmacokinetics was inconclusive due to the limited dose range investigated. The results from this study indicate that the BPI molecules are cleared rapidly upon i.v. administration; thus, an alternative route of administration such as s.c. injection may be more favorable. Recently, s.c. injection of BPI molecules has been shown to be more than or as effective as i.v. injection; thus, the s.c. route will be more desirable for delivering the BPI molecules. Pharmacokinetic studies of BPI molecules upon s.c. injection with various formulations, including nanoparticle delivery methods, are being investigated.

This study shows that modification of the linker can affect in vivo properties of the BPI molecules, especially in reducing side effects. Modification of Acp-G-linker in Ac-PLP-BPI-NH₂-2 to a more hydrophilic PEG6-linker with a similar length in Ac-PLP-BPI-PEG6 lowers the
hypersensitivity reaction of the peptides. It was previously shown that PLP-BPI molecules have lower hypersensitivity reactions than PLP_{139-151} peptide, which has the highest hypersensitivity reactions in this study (Table 2). Ac-PLP-BPI-NH\textsubscript{2}-2 and Ac-PLP-BPI-PEG6 have similar \textit{in vivo} activities in suppressing EAE in the animal model while Ac-PLP-BPI-PEG3 with a shorter linker has lower activity, indicating that the distance separation between the two peptides is important for peptide \textit{in vivo} activity.

Although short linear peptides generally lack the ability to crosslink adjacent IgE molecules on mast cells and basophils (Alexander et al., 2002), repeated injections of peptides have been shown to induce hypersensitivity in MS patients (Smith et al., 2005). Clinical implications of anaphylaxis are illustrated by the termination of a phase II clinical trial using an altered peptide ligand derived from MBP\textsubscript{85-99} to regulate MS because some of the patients developed systemic hypersensitivity (Bielekova et al., 2000; Kappos et al., 2000). It has been suggested that the peptide induces the production of anti-IgG1 and anti-IgE antibodies. The administration of anti-IgE antibodies can block myelin-peptide-induced anaphylaxis, suggesting that the hypersensitivity reaction is \textit{via} an IgE-dependent mechanism (Smith et al., 2005). The anaphylaxis reaction in animal model can be avoided by formation of altered peptide ligand (APL) of MOG\textsubscript{35-55} peptide with mutation of the Ala residue at positon 37 to Ala to produce MOG-Ala37. The MOG-Ala37 mutant does not bind to IgG1 from the serum of mice immunized with MOG\textsubscript{35-55}/CFA; this peptide suppressed EAE and, upon repeated injection, did not generate anaphylaxis reaction (Leech et al., 2007). Thus, this method is useful for designing BPI molecules that have no hypersensitivity reaction. In this study, the difference in hypersensitivity of PLP, Ac-PLP-BPI-NH\textsubscript{2}-2, and Ac-PLP-BPI-PEG6 molecules is possibly also due to the IgE
and IgG1 mechanisms; this hypothesis is still under investigation. We also observed that the administration of lower doses of BPI molecules (<100 nmol/injection) reduces hypersensitivity reactions, suggesting that induction of anaphylaxis by BPI molecules is dose-dependent. A similar observation was found in a clinical trial of APL in which lower doses of peptide administration were shown to reduce side effects of the peptide administration. Therefore, the effect of dose of BPI molecules on the sensitivity reaction is currently being investigated to find the optimal dose that is both low and well tolerated.

The possible mechanisms of action of BPI molecules are elucidated by measuring their effect on IL-17 production during the treatment of EAE. It is widely accepted that T cells that produce pro-inflammatory cytokines IL-17 (Th17) are the major contributor to the autoimmune pathogenesis of EAE or MS (Kasper et al. 2007). Elevated levels of IL-17 have been demonstrated in the cerebrospinal fluid and in brain lesions of patients with MS (Matusevicius et al., 1999; Lock et al., 2002; Hedegaard et al., 2008) and were shown to correlate with disease activity (Hedegaard et al., 2008). On day 12, the production of IL-17 is significantly higher in mice treated with BPI molecules (Ac-PLP-BPI-NH₂-2 or Ac-PLP-BPI-PEG6) than in PBS-treated mice. However, on day 35, the BPI-treated animals have lower IL-17 than the PBS-treated mice, suggesting that the BPI molecules have a long-term suppressive effect. Because both BPI molecules suppressed and ameliorated EAE effectively as indicated by the lower EAE scores, lower EAE incidence, and minimal loss in body weight, the only plausible explanation is that IL-17 up-regulation in the peptide-treated mice is due to the presence of the antigenic sequence in BPI molecules. Also, high levels of IL-17 in BPI-treated mice could be attributed to TGF-β production as a result of injection of soluble peptide. It is now known that TGF-β and IL-
6 play an important role in the induction of Th17 differentiation (Korn et al., 2009; Morishima et al., 2009). Interestingly, there was a significant decrease in IL-17 production at the end of the study among mice receiving either Ac-PLP-BPI-NH₂-2 or Ac-PLP-BPI-PEG6, further corroborating our earlier belief that the initial IL-17 response was due to the antigen used during priming. On the other hand, there was a significant increase in IL-17 production among PBS-treated mice on day 35, suggesting the possibility of an increased and/or an impending relapse of EAE. A better understanding of the up-regulation/down-regulation of IL-17 would be gained through studies carried out for periods much longer than 35 days as well as using priming agents other than PLP₁₃⁹-₁₅¹.

As mentioned previously, the central hypothesis is that the BPI molecules simultaneously bind to MHC-II and ICAM-1 of the immunological synapse at the surface of APC and selectively alter or suppress the activation of a subpopulation of autoreactive T cells in an antigen-specific manner. Thus, we anticipate that BPI should modulate only those immune responses involved in EAE while minimizing the nonspecific suppression of the entire immune system. Another BPI molecule called GAD-BPI had been shown to colocalize the MHC-II and ICAM-1 on the surface of APC (B cells) isolated from non-obese diabetes mice (Murray et al., 2007). However, how this colocalization event regulates and alters the commitment of T cells has not been fully elucidated. The mechanisms of action of BPI molecules could also be explained by invoking the mechanism of peptide vaccines as antigen-specific immunotherapy (SIT) (Fig. 5). When dendritic cells encounter insoluble antigen or complex molecules found on microbes such as bacteria and viruses, they are upregulated and mature, presenting the processed antigenic peptides using MHC-II molecules on their surface. As they mature, they upregulate the
costimulatory molecules required for full activation of T cells, thus leading to allergic and autoimmunity response upon recognition of the antigenic peptides by the T-cell receptors (TCR). This paradigm is based on findings that insoluble peptides can induce local inflammation (Shen et al., 2003) whereas soluble peptides can bind directly to dendritic cells (DCs) in lymphoid tissues (Santambrogio et al., 1999; Metzler et al., 2000) and may activate regulatory T cells (Dhodapkar et al., 2001; Menges et al., 2002). It has been suggested that the soluble peptide vaccine binds mostly to steady-state (immature) APC such as dendritic cells. Similarly BPI molecules bind to steady state APC, and upon binding of these loaded APC to naïve T cells, the T cells differentiate regulatory T cells (T-reg), which produce IL-10 (Larche and Wraith, 2005). The IL-10 producing T-reg cells suppress the T-cell activation via Th1 and/or Th17 mechanisms (Fig. 5). Our previous data showed that the production of TGF-β- and IL-10-producing CD4+CD25+ T cells were significantly increased in PLP-BPI-treated mice compared to PBS-treated mice; suggesting that the CD4+CD25+ regulatory T cells (T-reg) are in part responsible for the EAE-suppressive activity of BPI (Kobayashi et al., 2007, 2008). However, this does not satisfactorily explain why BPI was much more effective than PLP in suppressing EAE (Kobayashi et al., 2007) as well as differences in activities of various BPI derivates. Thus, it is also possible that the PLP-BPI molecule binds to mature dendritic cells via simultaneous binding to MHC-II and ICAM-1, which prevents the formation of the immunological synapse formation upon binding of mature dendritic cells to T cells (Fig. 5). This inhibition of the immunological synapse formation generates IL-4 producing Th2 cells as previously observed (Kobayashi et al., 2007). Thus, the BPI molecule acts via two different routes to suppress the T-cell activation
whereas PLP peptide alone acts via T-reg activation by binding to immature dendritic cells. Currently, these possible mechanisms are being investigated in more detail.

In conclusion, Ac-PLP-BPI-PEG6 effectively inhibited the onset, severity, and incidence of EAE, and its EAE-suppressive activity was comparable to that of Ac-PLP-BPI-NH₂-2. The Ac-PLP-BPI-PEG6-treatment has also been shown to lower hypersensitivity reactions compared to Ac-PLP-BPI-NH₂-2 and PLP treatment of EAE animals. Further alteration of the structure of BPI molecules may eliminate the hypersensitivity reaction. Currently, the mechanisms of action of BPI molecules and their dosing methods are also being investigated for efficacy optimization as well as lowering of their side effects. Finally, altering the structure and sequence of BPI molecules will lead to a more efficient and safer BPI-based therapy for MS.

Acknowledgement

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References


Footnotes:

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Figure Legends

**Figure 1.** The therapeutic efficacy of BPI derivatives in reversing EAE in the mouse model. The mice were immunized with PLP\textsubscript{139-151}/CFA followed by i.v. treatments up to a maximum of three injections with the BPI molecules starting on the day of disease onset as described in Materials and methods. **A**, Clinical EAE disease score. **B**, Change in body weight. Results are expressed as the mean ± S.D. (n = 10). There are significant differences between peptide-treated and PBS-treated groups in clinical disease scores; Ac-PLP-BPI-NH\textsubscript{2-2} (***p < 0.001), Ac-PLP-BPI-PEG6 (***p < 0.001), and Ac-PLP-BPI-PEG3 (**p < 0.001). Statistical values for the loss of body weight compared to PBS were as follows: Ac-PLP-BPI-NH\textsubscript{2-2} (***p < 0.001), Ac-PLP-BPI-PEG6 (***p < 0.001), and Ac-PLP-BPI-PEG3 (*p > 0.05).

**Figure 2.** *In vivo* EAE-suppressive activity of BPI derivatives in mouse EAE model. PLP\textsubscript{139-151}/CFA-immunized mice received intravenous injections of indicated peptide (100 nmol/mouse/day) on days 4 and 7. **A**, Clinical EAE disease score. **B**, Change in body weight. **C**, Incidence of disease. Results are expressed as the mean ± S.D. (n = 10). There are significant differences between BPI-treated and PBS-treated groups in clinical disease scores and loss of body weight; Ac-PLP-BPI-NH\textsubscript{2-2} (***p < 0.001), Ac-PLP-BPI-PEG6 (***p < 0.001). There was no significant difference (*p > 0.05) in EAE clinical scores and loss of body weight between scrambled peptide (Ac-PLP-BPI-PEG\textsubscript{SC}) and PBS groups. Also, no significant difference (*p > 0.05) between Ac-PLP-BPI-NH\textsubscript{2-2} and Ac-PLP-BPI-PEG6 was observed.
**Figure 3.** Production of interleukin 17 (IL-17) in serum *in vivo*. SJL/J female mice were immunized subcutaneously with PLP$_{139-151}$/CFA and injected intraperitoneally with pertussis toxin on days 0 and 2. Then, the mice received intravenous injections of indicated peptide (100 nmol/mouse/day) or PBS on days 4, 7, and 10. (A) Serum concentration of IL-17 determined on days 12 and 35 in Ac-PLP-BPI-NH$_2$-2-treated, Ac-PLP-BPI-PEG6-treated, PBS-treated, and unprimed mice using ELISA. Data are represented as mean ± S.D. (6 mice per group) and experiments were done in duplicate. Disease progression was evaluated by (B) EAE clinical disease score, (C) percent change in body weight, and (D) incidence of disease. There are significant differences between BPI-treated and PBS-treated groups in clinical disease scores and loss of weight; Ac-PLP-BPI-NH$_2$-2 (**p < 0.001), Ac-PLP-BPI-PEG6 (**p < 0.001). There was no significant difference (*p > 0.05) in EAE clinical scores and loss of body weight between Ac-PLP-BPI-NH$_2$-2 and Ac-PLP-BPI-PEG6.

**Figure 4.** Plasma concentration profiles of Ac-PLP-BPI-NH$_2$-2 after intravenous administration in Spraque-Dawley rats *in vivo*. Data are represented as mean ± S.D. with (n = 3) and in triplicate. Point marks represent actual plasma levels and the solid lines represent predicted plasma levels after fitting the raw data into compartmental models using commercial pharmacokinetic software.

**Figure 5.** Potential mechanism(s) of action of antigenic peptide or BPI molecule in the suppression of EAE. Mature dendritic cells present processed antigenic peptides to naïve T cells to generate Th1 and/or Th17 cells. BPI molecule binds simultaneously to MHC-II and ICAM-1.
on mature dendritic cells and prevents the formation of the immunological synapse. As a result, BPI molecule inhibits the formation of Th1 and generates Th2 cells. Immature dendritic cell can also present soluble antigenic and BPI molecule to naïve T cells to produce IL-10-producing T-reg. Produced IL-10 suppresses the generation of Th1 and Th17 cells, resulting in the suppression of the disease.
Table 1: List of peptides used in the present study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>PLP&lt;sub&gt;139-151&lt;/sub&gt;</td>
<td>HSLGKWLGHPDKF</td>
</tr>
<tr>
<td>LABL (CD11a&lt;sub&gt;237-246&lt;/sub&gt;)</td>
<td>ITDGEATDSG</td>
</tr>
<tr>
<td>Ac-PLP-BPI-NH&lt;sub&gt;2&lt;/sub&gt;-2</td>
<td>Ac-HSLGKWLGHPDKF-(AcpGAcpgAcpg)&lt;sub&gt;2&lt;/sub&gt;-ITDGEATDSG-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Ac-LABL-PLP-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Ac-ITDEATDSG-AcpGAcpgAcpg-HSLGKWLGHPDKF-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Ac-PLP-BPI-PEG3</td>
<td>Ac-HSLGKWLGHPDKF-(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;O)&lt;sub&gt;3&lt;/sub&gt;-ITDGEATDSG-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Ac-PLP-BPI-PEG6</td>
<td>Ac-HSLGKWLGHPDKF-(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;O)&lt;sub&gt;3&lt;/sub&gt;-G-(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;O)&lt;sub&gt;3&lt;/sub&gt;-ITDGEATDSG-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Ac-PLP-BPI-PEG6&lt;sub&gt;SC&lt;/sub&gt;</td>
<td>Ac-SLKHGGLWPHKDF-(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;O)&lt;sub&gt;3&lt;/sub&gt;-G-(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;O)&lt;sub&gt;3&lt;/sub&gt;-TDGITSGEDA-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

BPI is composed of antigen epitope peptide (PLP<sub>139-151</sub>), varying spacers, and LABL peptide (CD11a<sub>237-246</sub>); where Acp in the spacer represents ε-aminocaproic acid. The peptides are capped at both ends, i.e., N-terminal acetylated (Ac-) and C-terminal amidated (-NH<sub>2</sub>).

Table 2: Incidence of anaphylactic response upon peptide challenge

<table>
<thead>
<tr>
<th>Treatment on days 4 and 7</th>
<th>Peptide Challenge on day 35</th>
<th>Incidence of anaphylaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>PLP_{139-151}</td>
<td>13/15 (87%)</td>
</tr>
<tr>
<td>Ac-PLP-BPI-NH$_2$-2</td>
<td>Ac-PLP-BPI-NH$_2$-2</td>
<td>9/20 (45%)</td>
</tr>
<tr>
<td>Ac-PLP-BPI-PEG6</td>
<td>Ac-PLP-BPI-PEG6</td>
<td>4/18 (22%)</td>
</tr>
<tr>
<td>Ac-PLP-BPI-PEG6$_{SC}$</td>
<td>Ac-PLP-BPI-PEG6$_{SC}$</td>
<td>0/19 (0%)</td>
</tr>
</tbody>
</table>

PLP_{139-151}/CFA-immunized mice treated with vehicle or peptide (100 nmol/100 µl) received intravenous injection of the indicated peptide on day 35. Incidence of anaphylactic response was determined as described in Materials and Methods.
Table 3: Summary of pharmacokinetic parameters of Ac-PLP-BPI-NH₂-2 after intravenous administration. Data are represented as mean ± S.D. (n = 3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>5 μmol/kg</th>
<th>1.8 μmol/kg</th>
<th>0.9 μmol/kg</th>
<th>0.3 μmol/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>nmoL*h/mL</td>
<td>41.35 ± 3.20</td>
<td>22.73 ± 1.84</td>
<td>9.97 ± 2.88</td>
<td>6.55 ± 0.24</td>
</tr>
<tr>
<td>C_max</td>
<td>nmol/mL</td>
<td>116.05 ± 24.92</td>
<td>69.59 ± 19.69</td>
<td>18.17 ± 4.50</td>
<td>3.84 ± 1.03</td>
</tr>
<tr>
<td>α</td>
<td>h⁻¹</td>
<td>7.08 ± 1.70</td>
<td>7.88 ± 2.23</td>
<td>10.17 ± 2.46</td>
<td>4.69 ± 2.83</td>
</tr>
<tr>
<td>β</td>
<td>h⁻¹</td>
<td>0.36 ± 0.15</td>
<td>0.26 ± 0.19</td>
<td>0.29 ± 0.06</td>
<td>0.23 ± 0.07</td>
</tr>
<tr>
<td>A</td>
<td>nmoL/mL</td>
<td>106.71 ± 25.82</td>
<td>65.85 ± 19.69</td>
<td>15.99 ± 3.71</td>
<td>2.51 ± 0.95</td>
</tr>
<tr>
<td>B</td>
<td>nmoL/mL</td>
<td>9.34 ± 4.24</td>
<td>3.74 ± 2.62</td>
<td>2.18 ± 0.81</td>
<td>1.34 ± 0.49</td>
</tr>
<tr>
<td>Cl</td>
<td>L/h/kg</td>
<td>0.12 ± 0.04</td>
<td>0.08 ± 0.01</td>
<td>0.01 ± 0.03</td>
<td>0.05 ± 0.00</td>
</tr>
<tr>
<td>V_d</td>
<td>L/kg</td>
<td>0.24 ± 0.07</td>
<td>0.25 ± 0.12</td>
<td>0.32 ± 0.11</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
<td>V_initial</td>
<td>L/kg</td>
<td>0.04 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>Half-life</td>
<td>h</td>
<td>2.17 ± 0.86</td>
<td>3.55 ± 2.06</td>
<td>2.73 ± 0.63</td>
<td>3.28 ± 0.91</td>
</tr>
</tbody>
</table>
Fig. 1A

The figure shows a line graph plotting EAE clinical score against days. The x-axis represents days, ranging from 0 to 35, and the y-axis represents EAE clinical score, ranging from 0 to 3. The graph compares different treatments:

- **PBS** represented by diamonds
- **Ac-PLP-BPI-PEG3** represented by circles
- **Ac-PLP-BPI-PEG6** represented by triangles
- **Ac-PLP-BPI-NH2** represented by squares

The graph illustrates the clinical score over time for each treatment group, with error bars indicating variability.
Fig. 3A

![Bar chart showing mouse IL-17 levels for different peptides and serum concentrations.]

- **PBS**
- **Ac-PLP-BPI-PEG6**
- **Ac-PLP-BPI-NH2-2**
- **Non-immunized**

**Y-axis:** Mouse IL-17 levels (pg/mL)

**X-axis:** Peptide

- **Serum conc. on Day 12**
- **Serum conc. on Day 35**
Fig. 3B

![Graph showing EAE clinical score over days for different treatments: PBS, Ac-PLP-BPI-PEG6, and Ac-PLP-BPI-NH2-2.](chart.png)
Fig. 4

Plasma conc. of Ac-PLP-BPI-NH2-2 (pmol)

Time (min)

- 5000 nmol/kg dose
- 1800 nmol/kg dose
- 900 nmol/kg dose
- 300 nmol/kg dose