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Inhibition of cell adhesion and immune responses in the mouse model of collagen-induced arthritis with a peptidomimetic that blocks CD2-CD58 interface interactions

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Abstract

CD2 and CD58 are two important co-stimulatory molecules involved in generating the signal II required for normal immune signaling. However, this interaction can be targeted to be of benefit in cases of abnormal immune signaling seen in autoimmune diseases. Our objective in this study was to design a peptidomimetic (compound 7) based on a β -strand structure of the adhesion domain of CD2 protein to inhibit CD2-CD58 protein-protein interaction and its effect on immunomodulation in the collagen-induced arthritis (CIA) model. The ability of compound 7 to bind to CD58 protein was assessed using flow cytometry. The effect of compound 7 on modulating the immune response was evaluated in an autoimmune disease using CIA in mice. The stability of compound 7 was evaluated in mouse serum using mass spectrometry. Antibody (Ab) binding inhibition studies suggested that compound 7 binds to CD58 protein. Compound 7 was successful in modulating immune responses when administered in the CIA mouse model along with reducing anti-collagen Ab levels and decreasing the level of interferon gamma (IFN- γ) relative to control treatments. Compound 7 was found to be non-immunogenic and stable in mouse serum up to 48 h. Results suggest that compound 7 can serve as a lead compound for immunomodulation, and could be a therapeutic agent for the autoimmune disease rheumatoid arthritis (RA).

Keywords

peptidomimetic; immunomodulation; rheumatoid arthritis; co-stimulatory molecules; protein-protein interaction

INTRODUCTION

Three major families of cell surface receptor molecules that provide signaling for co-stimulation include the B7-CD28 superfamily, lymphocyte associated antigen-1 (LFA-1)

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and intercellular adhesion molecule-1 (ICAM-1), and the CD2 superfamily¹. CD58 on the surface of the antigen-presenting cells (APC) binds to CD2 on the T-cell surface following T-cell receptor (TCR) engagement. Specifically, the interaction of CD2 with CD58 enhances the TCR-peptide-major histocompatibility complex (pMHC) affinity by 100-fold. Upon interaction of CD2 with CD58, the cytoplasmic domain of CD2 initiates a cascade of signals to enhance interleukin-2 (IL-2) production that promotes T-cell survival²⁻⁶. In contrast, TCR signaling in the absence of co-stimulation can have a negative regulatory effect on T-cell activation. Therapeutic strategies that are developed to block the co-stimulatory pathways include the use of monoclonal antibodies, small molecule or peptide inhibitory compounds, and fusion proteins. Blocking or modulating CD2-CD58 signaling has been shown to have therapeutic utility for dampening immune responses^{7,8}. Rheumatoid arthritis (RA) is an autoimmune disorder that is inflammatory in nature and affects multiple joints (polyarthritis). During the progression of the disease, cartilage destruction caused by over recruitment of T-lymphocytes occurs at the joints. It has been postulated that, in the pathology of RA, there is overexpression of CD58 within affected tissues that provides an abundance of CD2 signaling to local T cells⁹. Hence, targeting the CD2-CD58 adhesion and co-stimulation signal for therapeutic intervention is beneficial in the treatment of RA¹⁰.

Our interest is to design peptides and peptidomimetics that inhibit or modulate CD2-CD58 interaction and suppress immune responses in the collagen-induced arthritis (CIA) mouse model. In our earlier studies, we have reported that peptides designed from CD2 protein inhibit cell adhesion and modulate immune response (Figure 1)¹¹⁻¹⁵. In the present work, we extend our studies to a peptidomimetic with a dibenzofuran moiety (DBF) (compound 7). Peptides with a dibenzofuran moiety that induces β -hairpin structure have been reported by other research groups^{16,17}. Such peptidomimetics are useful because they often show increased stability against enzymatic degradation¹⁸⁻²⁰. Compound 7 has been designed using the CD58 binding domain of CD2 protein based on our previous structure-activity relationship studies and has a β -strand structure that is stabilized by DBF moiety (Figure 2). In addition to inducing a β -strand structure, DBF also mimics a beta turn. The sequence of the CD2 adhesion domain was reversed in compound 7 to exhibit potential cell adhesion activity¹⁵. Our results show that compound 7 suppressed the progress of RA in a murine CIA mode. This suppression was accompanied by a reduction in collagen antibody levels and inflammatory cytokine production.

MATERIALS AND METHODS

Materials

All cells and media were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). FITC labeled antibody CD58 (FITC CD58Ab) was purchased from BD Biosciences (San Jose, CA). Flow cytometry studies were performed on BD FACS Calibur (BD Biosciences, San Jose, CA). Type II collagen, Complete Freund's Adjuvant (CFA) and incomplete Freund's adjuvant were purchased from Chondrex, Inc. (Redmond, WA). CellTiter-Glo assay kit was purchased from Promega, Inc. (Springfield, MO). Concanavalin A was purchased from GE Healthcare (Pittsburg, PA). The BD bio-array kit for cytokine levels determination was purchased from BD Biosciences. Immulon II plates

were purchased from Dynex technologies (VA). Tetramethyl benzidine was obtained from Kirkegard and Perry labs (Gaithersburg, MD). 4-(Fmoc-2-aminoethyl)-6-dibenzofuranpropionic acid was from Chem-Implex International (Wood Dale, IL) and all the amino acids and chemical reagents for peptide synthesis were from Applied Biosystems (Lifetechnologies, Grand Island, NY).

Synthesis of compound 7

Compound 7 was synthesized using standard solid-phase Fmoc chemistry²¹ using 2-chlorotriyl chloride resin (3 g, 0.89 mmol/g). After allowing the resin to swell in dichloromethane, it was then loaded with a solution of Fmoc-Ile-OH and *N,N*-diisopropylethylamine (DIEA) in 30 mL of dichloromethane. The mixture was agitated for 2 h, then filtered and washed with *N*-methyl-2-pyrrolidone (NMP) (30 mL, 6 × 30 sec), dichloromethane/methanol/*N,N*-diisopropylethylamine (DCM/MeOH/DIEA) (80:15:5, 2 × 15 min), NMP (5 × 30 sec), and dichloromethane (5 × 30 sec). The resin was dried under vacuum overnight and then deprotected with 30 mL of 20% piperidine/NMP (2 × 5 min). The remainder of the peptide sequence was synthesized using an automatic peptide synthesizer (Tribute Synthesizer). All amino acids (including DBF) were coupled using 5 equivalents of Fmoc-protected amino acid and 5 equivalents of 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU) dissolved in 2 mL of a 0.4 M solution of 4-methylmorpholine in NMP. The amino acid solution was bubbled on the instrument for 2 min before it was added to the resin after which the resin was shaken for 30 min. The resin was then washed with DMF (5 × 30 sec) and then deprotected with 20% piperidine/NMP (2 × 5 min). This sequence was repeated for the remaining amino acids in the sequence using the synthesizer. After the last amino acid was added to the peptide, the final Fmoc protecting group was removed. The peptide was cleaved from the resin by adding 2,2,2-trifluoroethanol:dichloromethane (2 mL, 1:1) to the resin and agitating it for 3 h. The cleavage step was repeated 3 times. Cyclization of the peptide was performed after the cleavage of the peptide from the resin. The cleavage solutions were then combined, and the solution was evaporated at room temperature under a gentle stream of nitrogen. The remaining solid was dissolved in 100 mL of tetrahydrofuran (THF)/DMF (4:1) to which was added 3-hydroxy-3*H*-1,2,3-triazolo[4,5-*b*]pyridinato-*O*-tri-1-pyrrolidinyl-phosphorus hexafluorophosphate (PyAOP) (4 eq., 104 mg) and DIEA (7 eq, 70 µL). The solution was agitated for 2 h after which the solvent was evaporated under vacuum to yield a solid. The crude cyclic peptide was then treated with a deprotection cocktail (4 mL, trifluoroacetic acid (TFA):water:triisopropylsilane (TIPS), 95:2.5:2.5) for 3 h. The peptide solution was poured into a 50 mL centrifuge tube, and the peptide was precipitated with 30 mL of cold diethyl ether. The tube containing the peptide was centrifuged and the supernatant was removed. The crude peptide was dissolved in 0.1% TFA/water, and lyophilized to obtain a white powder as the crude peptide. For *in vivo* studies, compound 7 was custom synthesized by Aroz Technologies, LLC (Cincinnati, OH). Compound 7 was purified by HPLC, and the purity of the final product was determined using analytical HPLC, electrospray mass spectrometry (ESI-MS). High-resolution mass spectrometry data were obtained. The calculated exact mass of compound 7 was 1014.4334 and the *m/z* obtained from experimental high-resolution mass spectrometry data was

1014.4339 (Supporting Information). A detailed NMR structure of compound 7 has been reported¹⁵.

Competitive binding studies

The ability of compound 7 to bind to CD58 on Caco-2 cells was evaluated using FITC-CD58Ab in a competitive binding experiment and using flow cytometry and fluorescence plate reader assay in two different sets of experiments. Details of the procedures were published elsewhere²². Briefly, Caco-2 cells were distributed in 7 different vials and 50 μ L of FITC-labeled anti-CD58 with or without 100 μ L of compound 7 was added at different concentrations. Cells with fluorescently labeled anti-CD58 were incubated at 4 °C for 30 min and then subjected to $720 \times g$ at room temperature for 10 min, resuspended in 4–5 mL of PBS, and transferred into flow cytometry tubes on ice. The samples were analyzed using a BD FACS flow cytometer. For the fluorescence assay using a plate reader, cells were incubated with FITC-CD58Ab (pre-diluted, 20 μ L) and/or compound 7 for 1 h after they reached confluency. The plate was washed 3 times after incubation, and fluorescence was read using a microplate reader at excitation λ 485 nm and emission λ 528 nm. Data were obtained as a set of triplicates. Fluorescence from the blank was subtracted for the final representation.

In vivo studies

Female DBA/1 mice 6–7 weeks old were obtained from Harlan Laboratories (Indianapolis, IN) and maintained in the ULM School of Pharmacy vivarium. Animal studies were conducted in strict accordance with good animal practice as defined by NIH guidelines, and all animal work was approved by the IACUC, University of Louisiana at Monroe (ULM). Animals were divided into six groups with eight mice in each group. The groups were G1-control, G2-arthritic mice, G3-arthritic mice treated with control peptide, G4-arthritis mice treated with compound 7 at a concentration of 1 mg/kg, G5-arthritis mice treated with compound 7 at a concentration of 0.5 mg/kg, and G6-mice treated with compound 7 at a concentration of 0.25 mg/kg. The induction of arthritis in the DBA-1 strain of mice was performed through the intradermal route of administration using type II collagen formulation (CII) emulsified in CFA following published procedures^{23–25}. Emulsion containing 3 mg/mL of CII (100 μ L) was injected intradermally at the base of the tail for the first immunization. A booster injection of CII emulsified (with incomplete Freund's adjuvant (IFA)) was given on day 21 of the study^{23,24}. The peptides were dissolved in PBS and treatment was started on 22nd day through intravenous tail-vein injection. A total of 5 injections of compound 7 and control peptide (1 mg/kg) in PBS were given on alternate days. The monitoring of mice was started on day 21 to determine the onset and severity of the disease. Wrist and ankle joints were inspected on alternate days beginning on the 21st day post-immunization. The visual appearance of the limbs was graded on a scale of 0–4^{24,26}. A Mann Whitney test using graph pad prism was performed to determine the statistical difference among the different groups for days 30 to 46. A one-way ANOVA was performed to determine the statistical significance among various treatment groups.

Histopathology analysis

Histopathology for disease progression and suppression was performed on samples obtained from hind limbs in 4 mice from each group on day 57 after mice were sacrificed. Hind limbs were fixed in 10% formalin and decalcified in EDTA for 60 days. Decalcification was verified by adding oxalic acid and observing the precipitate. Sections were stained with haematoxylin and eosin (H & E).

T-cell proliferation assay

This assay required the immunization of DBA-1 female mice with 100 µg of native type II collagen emulsified with CFA 12 days prior to the actual day of assay. APC were generated from naïve mice without type II collagen injection. Spleen cells were isolated from the immunized and control mice. T cells were filtered using a pre-washed nylon column and published procedures²². Spleens of normal mice were collected in a similar manner. 0.1 mL (4×10^6) of enriched T cells, 0.1 mL (1×10^7) of APC, and 10–20 µL of 1 mg/mL arthrogen-CIA T-cell grade type II collagen were seeded in 96-well flat bottom plates and incubated at 37 °C for 3 days in a CO₂ incubator. After harvesting the cells, compound **7** was added at different concentrations. Inhibition studies were also carried out with the addition of anti-CD4 and anti-CD8 antibodies to culture wells. Similar experiments were performed using control peptide^{26,27}. Concanavalin (Con A) was used as a positive control whereas medium containing only cells was used as a negative control.

Cytokine analysis

The levels of different cytokines from the blood of mice were determined using the BD Cytometric Bead Array Th1/Th2 kit. The test samples were diluted by 1:100 dilution factors using the assay diluents and were mixed properly. 50 µL of the mouse Th1/Th2 PE detection reagent was added to all assay tubes, and the tubes were further incubated for 2 h at room temperature protected from light. After incubation, 1 mL of wash buffer was added in each tube and centrifuged at $200 \times g$ for 5 min. The supernatant was discarded and 300 µL of wash buffer was added to each tube to resuspend the bead pellet. Finally, mouse Th1/Th2 cytokine data were analyzed using flow cytometry.

Measurement of anti-collagen Ab levels

Serum was collected from blood samples by centrifugation at 2000 rpm. Levels of anti-collagen antibodies were determined using this serum in various groups of mice. Immulon II plates were coated with bovine type II collagen (1 µg/mL, 0.1 mL/well) in carbonate/bicarbonate buffer and kept at 4 °C overnight. The next day they were washed with PBS containing 0.05% Tween-20 and blocked with 1% BSA for 30 minutes. Serum samples were diluted 50-fold in 1% BSA followed by threefold serial dilutions. These samples were added in triplicate into each well (50 µL) and incubated for 2 h at room temperature. Bound anti-collagen Abs were determined using HRP-labeled goat anti-mouse IgG. Tetramethyl benzidine (100 µL/well) was added to each well and allowed to develop color before stopping the reaction with 1 M sulfuric acid. Absorbance was read at 450 nm with background subtraction at 630 nm²⁸. Readings were in triplicate and the experiment was repeated twice.

Stability studies

200 μ L of stock solutions of compound **7** was treated with 1 mL of purchased serum (Sigma-Aldrich, St Louis, MO). At different time points ranging from 0 min to 48 h, 100 μ L of the above sample was withdrawn and treated with 500 μ L of chilled acetonitrile for compound **7** extraction. This sample was vortexed followed by centrifugation at $13,000 \times g$ for 15 min. The supernatant was passed through a SEP-PAK C18 column and lyophilized²⁹. These samples were analyzed by TOF-MS. The matrix was prepared as a 10 mg/mL solution of α -cyano-4-hydroxy-cinnamic acid (Sigma) in 2:1 water:acetonitrile containing 0.1% TFA by volume. A 5 μ L aliquot of the sample solution was combined with 5 μ L of the matrix solution. 1 μ L of this mixture was spotted onto a MALDI target plate and allowed to dry. MALDI-TOF MS measurements were performed on Ultraflextreme (Bruker Daltonics, Billerica, MA). Leuprolide (Sigma-Aldrich) was used as an internal standard (molecular weight 1209)³⁰. The relative intensity of the peak vs. time was plotted.

RESULTS

Synthesis of compound **7**

Compound **7** was synthesized by solid-phase peptide synthesis.²¹ The compound was cleaved from the resin using a TFA cocktail and purified by HPLC with a C-18 column. The product was analyzed for purity using analytical HPLC and mass spectrometry. The structure of compound **7** was characterized by circular dichroism (CD) and NMR as previously reported¹⁵.

Competitive binding of compound **7** to CD58 expressed by Caco-2 cells in the presence of FITC-labeled anti-CD58

Compound **7** was designed from the surface epitope of the CD2 protein involved in the binding of CD58. It was presumed that compound **7** will bind to CD58 and inhibit the protein-protein interactions of CD2 and CD58, resulting in cell adhesion inhibition between T cells and Caco-2 cells. The binding of compound **7** to CD58 was evaluated using Caco-2 cells that express CD58 in the presence of FITC-CD58Ab using a fluorescence assay and flow-cytometry analysis. Expression of CD58 protein on Caco-2 cells was verified by binding of FITC-CD58Ab. Compound **7** was incubated at increasing concentrations (0.05 to 10 μ M) in the presence of FITC-CD58Ab, and relative fluorescence was measured using a plate reader in the fluorescence assay. The fluorescence intensity of FITC-CD58Ab was measured; it was found that in the presence of compound **7**, CD58Ab binding was decreased substantially even at low concentration of 0.05 μ M. This indicated the competitive binding of compound **7** to CD58 protein on Caco-2 cells (Figure 3). In the second set of experiments using flow-cytometry, similar results were obtained. Assessment of Caco2 cells labeled with FITC-CD58Ab and incubated with increasing concentrations of compound **7** (10 to 200 μ M), showed a shift of labeled cells toward the left in the histogram compared to the cell population that was labeled with FITC-CD58Ab, suggesting the competitive binding of compound **7** to CD58. At 200 μ M of compound **7**, nearly 85% inhibition of antibody binding was observed (Figure 4).

Compound 7 suppresses CIA in DBA/1 mice

The *in vivo* effect of compound 7 to suppress the progression of arthritis was evaluated in an autoimmune disease model, collagen-induced arthritis (CIA) in DBA/1 mice. DBA/1 mice were induced with arthritis using type II collagen. After induction of arthritis, compound 7 was administered through i.v. injection starting on day 22; treatment was then continued for 5 doses administered on alternate days. To assess the disease progression, scoring system by visual appearance of limbs and digits was used^{23,31}. Compound 7 at 1 mg/kg, 0.5 mg/kg, and 0.25 mg/kg showed significant reduction in arthritis score from days 40 to 46 as compared to control peptide and untreated mice group (Figure 5). The Mann Whitney U Test was applied using a 95% confidence interval to determine the statistical significance and variability within the groups. There was a significant difference (with $P < 0.05$) between the control peptide vs. compound 7 treated groups with 0.25, 0.5, and 1 mg/kg treatment. However, when we compared the treatment groups at different doses of compound 7, there were no significant differences observed between mice treated with 0.5 mg/kg and 1 mg/kg of compound 7. The mice did not show any clinical signs of illness such as anorexia, hunched posture, ruffled fur, and/or lethargy during dosing or prior to the onset of observed clinical arthritis.

Histopathology analysis of tissue samples

Histopathology analysis of tissue samples of limbs was carried out using H&E stained slides from different groups of mice. In the normal animals, it was seen that surfaces of cartilage were smooth and intact. Articular cartilaginous surfaces of both bones were intact, with a smooth and clear joint space as seen in Figure 6A. This result correlates with the visual scoring of the hind limbs of the normal group of mice where there was no inflammation seen at the joints. The H & E stain (original 100× magnification) of the arthritis group of phalangeal joints with severe (Grade 4) arthritis indicated the cartilaginous erosion of joint surface and pannus formation around the cartilage. There was a significant infiltration of mononuclear cells surrounding the pannus formation as seen in Figure 6B. This observation was supported by the fact that mice in this group had severe inflammation and reddening with all digits visually swollen during disease progression. In the case of compound 7 (0.5 mg/kg) treated mice, H & E staining of the hind paw indicated clear joint space with a normal cartilage interface. Though connective tissue surrounding the joint was heavily infiltrated with mixed inflammatory cells, there was a reduction in pannus formation as shown in Figure 6C. These results suggest that compound 7 could suppress the progression of arthritis in the CIA model.

Compound 7 inhibited the production of anti-collagen Abs

The degree of severity of arthritis in mice can be evaluated by the quantity of CII-specific antibody in the serum of a mouse that develops CIA. The autoimmune response to CII can be evaluated by measuring the quantity of the CII-specific antibody in the sera of the mice. Serum samples were analyzed at the termination of the study in different groups of mice to evaluate whether compound 7 can inhibit the production of anti-CII Abs. The results suggested that compound 7 was able to reduce the amount of CII in mice that developed

arthritis compared to the control group. This reduction in CII (Figure 7A) suggested that inhibition of adhesion leads to modulation of the immune response.

Compound 7 reduces level of cytokines in serum of mice

Elevated serum levels of inflammatory cytokines such as TNF- α and IFN- γ and chemokines like IL-5 are hallmarks of rheumatoid arthritis development and CIA. In RA patients, these cytokines have been found to be at higher levels than in normal patients and contribute to further progression of bone and cartilage destruction. We wanted to determine whether compound 7 could lower the levels of IFN- γ by blocking CD2-CD58 interaction. It was observed that compound 7 at 1 mg/kg was able to reduce the levels of IFN- γ compared to those in control peptide treatment and untreated arthritic groups of mice. This indicated that by inhibiting protein-protein interaction we could lower the levels of cytokines in blood sera of DBA/1 mice (Figure 7B).

Compound 7 immunogenicity

Peptides that are not endogenous to the body can trigger both cellular and humoral immune responses when administered. We therefore wanted to evaluate whether the peptides administered into mice are immunogenic or not. This was determined by measuring the response of isolated splenocytes from primed mice to the peptides *in vitro*. It was observed that varying doses of compound 7 did not generate any measurable T-cell memory response in cultured splenocytes. Compound 7 at either dose (1 or 0.25 μ M) did not induce proliferation of t-cells as compared to controls. Con A was used as a positive control and indicated proliferation of cells more than 40 times that of compound 7-treated cells (Figure 7C).

Stability of Compound 7 in mouse serum

To prevent enzymatically facilitated peptide degradation, several strategies based on the chemical modification of the peptides are generally applicable^{32–34}. Compound 7 was incubated in mouse serum, and its stability was evaluated using mass spectrometry. Since *in vivo* studies were conducted in a mouse model, use of mouse serum is appropriate. The stability of compound 7 was assessed by comparing the intensity of the peak in the mass spectrum³⁰ with that of the freshly prepared leuprolide that was used as internal standard. The recovery of compound 7 from mouse serum was detectable at all time points, and the half-life for compound 7 in mouse serum was estimated to be around 2 h (Figure 8). More accurate quantitative determination of the actual amount of peptide degraded at different time points will be performed in future studies using HPLC and quantitating the peak area under the curve.

DISCUSSION

Compound 7 described in the present study was derived from the adhesion region (F and C strands) of CD2 protein and has been shown previously to block the adhesion interaction between T cells and epithelial cells by inhibiting CD2-CD58 interaction^{6,22}. The β -turn/hairpin in the structure of compound 7 was stabilized by a β -turn mimicking dibenzofuran moiety¹⁶. Competitive binding studies using fluorescence-labeled antibodies suggested that

compound 7 binds to CD58, presumably to the adhesion domain of CD58, and inhibits the interaction between CD2-CD58. In the fluorescence assay using Caco-2 cells (Figure 3), compound 7 exhibited inhibition of antibody binding at as low as 0.05 μM concentration. Compared to 0.05 μM of compound 7, 0.5 and 10 μM concentrations of compound 7 exhibited higher inhibition of antibody binding. However, there was no clear dose response of competitive binding of compound 7 to CD58 protein on Caco-2 cells. Since antibody to CD58 binds to CD58 protein on cells with high specificity at nanomolar concentration, it may not be possible to completely inhibit FITC-antiCD58 binding to CD58 by compound 7. We believe that this might be the reason for the results observed (Figure 3) for compound 7. Our ultimate aim was to design a peptidomimetic which can inhibit CD2-CD58 PPI in the pathogenesis of RA. Compound 7 was able to suppress the progression of arthritis at all the doses, i.e., 0.25, 0.5, and 1 mg/kg, respectively, indicating the ability of peptides to modulate the immune response in *in vivo* studies. The suppression of CIA³⁵ was confirmed by the visual arthritis scoring of the inflammation of the limbs as well as by histopathology analysis. Compound 7 was able to reduce the levels of IFN- γ compared to the levels in the arthritic group. Although we have not yet performed dose escalation studies for toxicity assessment, our observations from *in vivo* studies did not indicate any toxic effects of the compound in experimental animals. Compound 7 did not show any immunogenicity³⁶ when compared to the positive control con A. We can presume that the immunomodulation by compound 7 occurs by binding to the receptor protein and inhibiting protein-protein interactions of CD2-CD58/CD48. We have used a cyclization approach to improve the overall stability of the peptide while preserving its potency by retaining the important residues of the original adhesion domain sequence of CD2 protein. DBF moiety was added in compound 7, which imparted β -turn structure in the designed peptidomimetic. Along with β -turn constraints, cyclization of the peptide backbone helps to confer a structural constraint that reduces conformational flexibility³⁷. Stability studies indicated that the half-life of compound 7 in mouse serum was 2 h and that the compound could be detected in serum even at the 48 h time point (Figure 8). This clearly suggests that compound 7 was stable in circulation *in vivo* to exert its pharmacological action. More than 50% of the compound 7 was degraded in 4 h, and the amount of intact compound 7 reached a plateau around 4 h as seen in Figure 8. One possibility for the observed plateau after about 4 h is that compound 7 is binding to serum proteins^{38,39}. Most of the drug-like molecules/peptides bind to serum proteins. We have not investigated this in the present work. During extraction, the compound that is bound to serum protein may be released into organic medium, and we could detect that during mass spectrometry analysis.

CONCLUSIONS

In conclusion, a peptidomimetic compound 7 was designed to modulate the immune response. Cellular assays and *in vivo* studies indicated that compound 7 binds to the adhesion domain of CD58 protein and was able to suppress progression of CIA in the DBA/1 mouse model. Anti-CII antibody levels were also found to be reduced in mice treated with compound 7 as were the levels of IFN- γ . Thus, our studies demonstrate that protein-protein interactions between CD2-CD58 could be modulated by a peptidomimetic *in vivo*, and it could be therapeutically useful for treating autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. van der Merwe PA, Davis SJ. Annu Rev Immunol. 2003; 21:659–684. [PubMed: 12615890]
2. Hutchings NJ, Clarkson N, Chalkley R, Barclay AN, Brown MH. J Biol Chem. 2003; 278:22396–22403. [PubMed: 12690097]
3. Kanner SB, Damle NK, Blake J, Aruffo A, Ledbetter JA. J Immunol. 1992; 148:2023–2029. [PubMed: 1372020]
4. Martelli MP, Lin H, Zhang W, Samelson LE, Bierer BE. Blood. 2000; 96:2181–2190. [PubMed: 10979964]
5. Espagnolle N, Depoil D, Zaru R, Demeur C, Champagne E, Guiraud M, Valitutti S. Int Immunol. 2007; 19:239–248. [PubMed: 17220479]
6. Musgrave BL, Watson CL, Hoskin DW. J Interferon Cytokine Res. 2003; 23:67–81. [PubMed: 12744772]
7. Felix NJ, Suri A, Salter-Cid L, Nadler SG, Gujrathi S, Corbo M, Aranda R. Autoimmunity. 2010; 43:514–525. [PubMed: 20429850]
8. Stuart RW, Racke MK. Expert Opin Ther Targets. 2002; 6:275–289. [PubMed: 12223069]
9. Bierer BE, Burakoff SJ. FASEB J. 1988; 2:2584–2590. [PubMed: 2838364]
10. Hoffmann JC, Herklotz C, Zeidler H, Bayer B, Rosenthal H, Westermann J. Ann Rheum Dis. 1997; 56:716–722. [PubMed: 9496150]
11. Liu J, Li C, Ke S, Satyanarayanajois SD. J Med Chem. 2007; 50:4038–4047. [PubMed: 17658775]
12. Li C, Satyanarayanajois SD. Peptides. 2007; 28:1498–1508. [PubMed: 17689835]
13. Giddu S, Subramanian V, Yoon HS, Satyanarayanajois SD. J Med Chem. 2009; 52:726–736. [PubMed: 19123855]
14. Satyanarayanajois SD, Buyuktimkin B, Gokhale A, Ronald S, Siahaan TJ, Latendresse JR. Chem Biol Drug Des. 2010; 76:234–244. [PubMed: 20572813]
15. Gokhale A, Weldeghiorghis TK, Taneja V, Satyanarayanajois SD. J Med Chem. 2011; 54:5307–5319. [PubMed: 21755948]
16. Kaul R, Deechongkit S, Kelly JW. J Am Chem Soc. 2002; 124:11900–11907. [PubMed: 12358534]
17. Mayo KH, Dings RP, Flader C, Nesmelova I, Hargittai B, van der Schaft DW, van Eijk LI, Walek D, Haseman J, Hoye TR, Griffioen AW. J Biol Chem. 2003; 278:45746–45752. [PubMed: 12947097]
18. Hruby VJ, Cai M. Annual review of pharmacology and toxicology. 2013; 53:557–580.
19. Ko E, Liu J, Perez LM, Lu G, Schaefer A, Burgess K. J Am Chem Soc. 2011; 133:462–477. [PubMed: 21182254]
20. Marschutz MK, Zauner W, Mattner F, Otava A, Buschle M, Bernkop-Schnurch A. Peptides. 2002; 23:1727–1733. [PubMed: 12383859]
21. Coin I, Beyermann M, Bienert M. Nat Protoc. 2007; 2:3247–3256. [PubMed: 18079725]
22. Gokhale A, Kanthala S, Latendresse J, Taneja V, Satyanarayanajois S. Chem Biol Drug Des. 2013; 82:106–118. [PubMed: 23530775]

23. Brand DD, Latham KA, Rosloniec EF. Nat Protoc. 2007; 2:1269–1275. [PubMed: 17546023]
24. Williams RO. Methods Mol Med. 2004; 98:207–216. [PubMed: 15064442]
25. Taneja V, Behrens M, Mangalam A, Griffiths MM, Luthra HS, David CS. Arthritis Rheum. 2007; 56:69–78. [PubMed: 17195209]
26. de Vaucher RA, de Velho Gewehr CC, Correa AP, Sant’Anna V, Ferreira J, Brandelli A. Int J Pharm. 2011; 421:94–98. [PubMed: 21963470]
27. Morgan ME, Flierman R, van Duivenvoorde LM, Witteveen HJ, van Ewijk W, van Laar JM, de Vries RR, Toes RE. Arthritis Rheum. 2005; 52:2212–2221. [PubMed: 15986351]
28. Suchard SJ, Stetsko DK, Davis PM, Skala S, Potin D, Launay M, Dhar TG, Barrish JC, Susulic V, Shuster DJ, McIntyre KW, McKinnon M, Salter-Cid L. J Immunol. 2010; 184:3917–3926. [PubMed: 20190141]
29. Lord HL, Zhang X, Musteata FM, Vuckovic D, Pawliszyn J. Nat Protoc. 2011; 6:896–924. [PubMed: 21637206]
30. Pappa EV, Zompra AA, Spyrali Z, Diamantopoulou Z, Pairas G, Lamari FN, Katsoris P, Spyroulias GA, Cordopatis P. Biopolymers. 2011; 96:260–272. [PubMed: 20632397]
31. Khachigian LM. Nat Protoc. 2006; 1:2512–2516. [PubMed: 17406499]
32. Hess S, Ovadia O, Shalev DE, Senderovich H, Qadri B, Yehezkel T, Salitra Y, Sheynis T, Jelinek R, Gilon C, Hoffman A. J Med Chem. 2007; 50:6201–6211. [PubMed: 17983214]
33. Hunter HN, Jing W, Schibli DJ, Trinh T, Park IY, Kim SC, Vogel HJ. Biochim Biophys Acta. 2005; 1668:175–189. [PubMed: 15737328]
34. Tugyi R, Uray K, Ivan D, Feller E, Perkins A, Hudecz F. Proc Natl Acad Sci USA. 2005; 102:413–418. [PubMed: 15630090]
35. Kannan K, Ortmann RA, Kimpel D. Pathophysiology. 2005; 12:167–181. [PubMed: 16171986]
36. Kaumaya PT, Foy KC. Future Oncol. 2012; 8:961–987. [PubMed: 22894670]
37. Adessi C, Soto C. Curr Med Chem. 2002; 9:963–978. [PubMed: 11966456]
38. Vuignier K, Schappler J, Veuthey JL, Carrupt PA, Martel S. Anal Bioanal Chem. 2010; 398:53–66. [PubMed: 20454782]
39. Howard ML, Hill JJ, Galluppi GR, McLean MA. Comb Chem High Throughput Screen. 2010; 13:170–187. [PubMed: 20053162]

FIGURE 1. Schematic diagram showing the importance of adhesion molecules CD2-CD58 in immune response and inhibition of protein-protein interactions of CD2-CD58 by compound 7 and modulation of immune response.

FIGURE 2.
Structure of compound 7.

FIGURE 3.

Competitive binding of compound 7 with FITC-labeled antibody to Caco-2 cells expressing CD58 protein. Antibody binding to Caco-2 cells in the presence of compound 7 at different concentrations is shown. 1×10^6 cells/100 μL were redistributed into different wells of 96-well plates. After 24 h, attached cells were incubated with FITC-CD58Ab (pre-diluted, 20 μL) and/or compound 7 for 1 h. The plate was washed 3 times after incubation and fluorescence was read using a microplate reader at excitation λ 485 nm and emission λ 528 nm. Fluorescence from the blank was subtracted for representation. Data are from triplicate experiments.

FIGURE 4.

Competitive binding of compound **7** with FITC-labeled antibody to Caco-2 cells expressing CD58 monitored by flow cytometry (BD FACS Calibur). Cells were distributed in 7 different vials and added with 50 μ L of FITC-labeled anti-CD58 as positive control, 50 μ L of FITC-CD58Ab along with 100 μ L of compound **7** at concentrations of 10, 50, 100 and 200 μ M and controls. The shift in number of cells with or without FITC-CD58Ab was observed. Histograms show Caco-2 cells without peptide or antibody (more than 80% of the unstained cells are observed), cells with FITC-AbCD58 (there was a shift of cell population

to the right and nearly 75% of cells were stained), and cells with compound **7** and FITC-CD58Ab at compound **7** concentrations of 10, 50, 100, and 200 μ M, respectively. Note that in the presence of compound **7** more Caco-2 cells without antibody labeling (cell population shifted to left compared to cells + FITC antibody) were observed, suggesting the inhibition of FITC-CD58Ab to Caco-2 cells. A vertical dotted line was drawn for comparison of shifts of cells.

FIGURE 5.

Suppression of collagen-induced arthritis by compound 7. Treatment of mice by compound 7 shows suppression of collagen-induced arthritis (statistically significant) from days 40–46 at concentrations of 0.5 mg/kg and 1 mg/kg when compared to control peptide and vehicle. Scoring (0–4) was done according to the published procedure as described in the text for all the four limbs with a maximum score of 16. For plotting, the average score from 8 mice was used.

FIGURE 6.

Histopathological analysis of samples from hind limbs of normal, arthritic, and treated DBA/1 mice. Sections of paw (interphalangeal joints) and hind limb (tarsal joints) from 12 mice from different groups were examined. Representative sections from the tarsal joints of three mice are shown above.

(A) Normal tarsocrural joint (tibia and talus). Articular cartilaginous surfaces of both bones are intact and smooth (arrow heads) and the joint space is clear (*); H&E staining, 200 $\times$.

(B) Arthritic proximal intertarsal joint (talus and calcaneus) with grade 4 arthritis. Abundant pannus with thick articular cartilage in some areas (#) and cartilage ulceration in others

(thick arrows); periosteal bone proliferation (not shown); moderate inflammatory infiltrates consisting of histiocytes, plasma cells, and neutrophils. H&E staining, 100×.

(C) Proximal intertarsal joint (central and fourth tarsal bones) of mouse treated with compound **7** (0.5mg/kg). Severe pannus with moderate multifocal articular cartilage erosion and ulceration (thick arrows); bone lysis and destruction (thin arrows) with periosteal proliferation; histiocytic and neutrophilic infiltration. H&E staining; 100×

FIGURE 7.

A) Relative amounts of collagen II antibody in terms of absorbance was measured in different treatment groups. Serum was collected from blood samples of mice that developed arthritis and treatment groups as well as controls. These samples were added in triplicate into each well (50 μ L). Immulon II plates were coated with bovine type II collagen and incubated. After washing, bound anti-collagen Abs were determined using HRP-labeled goat anti-mouse IgG. Readings were in triplicate, and the experiment was repeated twice. The figure clearly indicates the difference between collagen II Ab levels in the arthritis group those in groups treated with compound **7**. Control peptide groups also showed higher collagen II Ab levels than the compound **7**-treated groups.

B) Levels of IFN- γ were measured in different groups of DBA/1 mice using BD biosciences bead array method and flow cytometry. The compound **7**-treated group at 1 mg/kg showed significant reduction in levels of IFN- γ compared to the arthritic group.

C) Cells from spleens of mice primed and challenged with compound **7** for antigenicity. DBA-1 female mice were immunized with 100 µg of native type II collagen emulsified with CFA 12 days prior to the actual day of assay. Antigen-presenting cells were generated from naïve mice without type II collagen injection. Cells were isolated from the immunized and control mice. After harvesting the cells, compound **7** was added at different concentrations. Plates were incubated for 45 min in a CO₂ incubator at 37 °C. This was followed by the final step of reading the fluorescence using a celltiter-glo (CTG) assay (Promega, Inc.) with a plate reader. Con A was used as positive control (p<0.05).

FIGURE 8.

Stability of compound 7 in serum by MALDI-TOF. Y-axis represents amount of intact compound 7 remaining. 200 μ L of stock solutions of compound 7 was treated with 1 mL mouse serum. At different time points ranging from 0 min to 48 h, 100 μ L of the above sample was withdrawn and treated with 500 μ L of chilled acetonitrile for compound 7 extraction. These samples were analyzed by TOF-MS. The spectra were acquired after calibration of the instrument with a peptide standard. A minimum of 500 laser shots per sample was used to generate each mass spectrum. Leuprolide was used as an internal standard against which the sample peaks were compared. Relative intensity is plotted with respect to time, and data are from triplicate experiments.