Published in final edited form as:

Mol Pharm. 2013 February 4; 10(2): 728–738. doi:10.1021/mp300539f.

Novel, Biocompatible, Disease Modifying Nanomedicine of VIP for Rheumatoid Arthritis

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Abstract

Despite advances in rheumatoid arthritis (RA) treatment, efficacious and safe disease-modifying therapy still represents an unmet medical need. Here we describe an innovative strategy to treat RA by targeting low doses of vasoactive intestinal peptide (VIP) self-associated with sterically stabilized micelles (SSMs). This spontaneous interaction of VIP with SSM protects the peptide from degradation or inactivation in biological fluids and prolongs circulation half-life. Treatment with targeted low doses of nano-sized SSM-VIP but not free VIP in buffer significantly reduced incidence and severity of arthritis in an experimental model, completely abrogating joint swelling and destruction of cartilage and bone. In addition, SSM associated VIP unlike free VIP had no side-effects on the systemic functions due to selective targeting to inflamed joints. Finally, low doses of VIP in SSM successfully downregulated both inflammatory and autoimmune components of RA. Collectively, our data clearly indicate that VIP-SSM should be developed to be used as a novel nanomedicine for the treatment of RA.

Keywords

inflammation; autoimmune disorders; phospholipids; micelles; nanomedicine; vasoactive intestinal peptide

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology that afflicts ~1% of the adult world population (~2 million patients in the United States). Despite remarkable advances in drug discovery and development, an efficacious and safe disease modifying therapy for RA represents an unmet medical need.

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Supporting information

Histograms of VIP-SSM efficacy against established CIA in mice; time plots of VIP-SSM and VIP biodistribution in CIA mice. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Targeting the two hallmark characteristics of RA - inflammation and autoimmunity has been suggested for the development of a mechanism-based therapy. Currently, monoclonal antibodies targeting pro-inflammatory cytokines are preferred remedies to treat RA.¹ However, recently many life-threatening side-effects such as systemic fungal infections, development of tuberculosis, and even in a few cases cardiac failure have been observed following their administration.²⁻⁵ Therefore, the long-term use of these biologics is limited.

An alternative therapeutic strategy to promote RA remission is shifting balance in favor of anti-inflammatory autoimmune response supporting immunologic homeostasis, specifically, targeting CD4+ T cells, central to the pathogenesis of RA, along with regulation of the inflammatory cell balance.⁶⁻⁸ Countless attention has been focused on shifting the response from type 1 helper T cells (Th1) to anti-inflammatory Th2 cell subset to promote RA remission.^{7, 8} In addition, the role of type 17 helper T cells (Th17) in regulation of RA pathogenesis gained recent attention.^{6, 9}

Vasoactive intestinal peptide (VIP), a mammalian 28 amino acid neuropeptide, has been shown to express a wide spectrum of functions controlling the innate homeostasis of the immune system.¹⁰⁻¹² The peptide action exerts through high affinity VIP receptors, which are known to be expressed on T-lymphocytes and various inflammatory cells.¹³⁻¹⁵ VIP has been shown to predominantly possess an anti-inflammatory action, such as shifting an immune reaction toward an anti-inflammatory Th2 type T cells response and downregulation of pro-inflammatory Th17 subset.¹⁶⁻¹⁸ The anti-inflammatory role of VIP has also been related to inhibition of macrophage functions such as COX-2/PGE2 system inhibition, phagocytosis, respiratory burst, and chemotaxis.^{19,20} Due to its various anti-inflammatory effects, the role of VIP in ameliorating RA on a pre-clinical model of collagen induced arthritis (CIA) was investigated.^{17,18} In these studies, multiple intraperitoneal administration of VIP, in relatively high doses, was able to significantly reduce clinical manifestation of experimental arthritis in rodents. Therapeutic action of VIP has been associated with downregulation of both inflammatory and autoimmune constituents of the disease.

However, in spite of these encouraging results, the clinical use of free VIP as-is is highly unlikely. This is due to multiple potential issues. First, VIP being a peptide has a short (few minutes) *in vivo* half-life,^{21,22} owing to its rapid enzymatic degradation.²³⁻²⁵ Therefore, repetitive high doses of VIP were compulsory to circumvent drawbacks of short circulation time of exogenously administered peptide to attain the therapeutic efficacy.^{17, 18} Second, the precipitous dose-dependent blood pressure drop is typically observed on administration of VIP as a free molecule.^{26,27} Thus, these serious downsides have precluded further clinical development of native/free VIP as an RA remedy.

Previously, we showed that intrinsic properties of PEGylated sterically stabilized phospholipid micelles (SSM) as a drug delivery vehicle enhanced stability, safety, and bioactivity of several peptide drug candidates.²⁸⁻³¹ In line, we also revealed that SSM promote remarkable conformational change of VIP from random coil to α -helix,^{32,33} which was shown to be preferable for interaction with the cognate receptors^{34,35} and linked to aqueous stability of the peptide.³⁶ Moreover, SSM-VIP particle size of ~15nm³⁷ offers an additional advantage in treatment of inflammatory diseases, providing a favorable balance between systemic clearance and extravasation via 'leaky' vasculature of inflamed sites, e.g. synovium of RA joints.³⁸

Given these issues, this study aimed to further optimize VIP-SSM and conduct studies on efficacy, distribution, and safety of the formulation *in vivo*. Here we demonstrated that optimized formulation of VIP-SSM preferentially localize in the inflamed joints,

significantly ameliorating clinical signs of experimental arthritis in mice at low VIP dose. Circulating levels of cellular modulators involved in the disease progression fully correlated with the clinical findings. Most importantly, SSM-VIP completely abrogated the peptide hypotensive side-effects. These properties warrant the further development of VIP-SSM as a novel safe disease-modifying nanomedicine for the treatment of RA.

EXPERIMENTAL SECTION

Materials

1,2-Distearoyl-*sn*-glycero-3-phosphatidylethanolamine-N- [methoxy (polyethyleneglycol)-2000] sodium salt (DSPE-PEG₂₀₀₀) was purchased from Northern Lipids Inc. (Vancouver, BC, Canada). Vasoactive intestinal peptide (VIP) was synthesized and purified by Research Resources Center of University of Illinois at Chicago (UIC). Radioactively labeled ¹²⁵I-VIP was acquired from Bachem (Torrance, CA). Human serum was purchased from Cambrex Laboratories (Santa Rosa, CA) and Bio-Spin P-30 columns from Bio-Rad (Hercules, CA). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from Amersham Biosciences (Hercules, CA) or R&D systems (Minneapolis, MN). All other reagents of analytical grade were purchased from Sigma-Aldrich Corp. (St. Louis, MO) or Fisher Scientific (Itasca, IL).

Sample preparation

SSM formulations were prepared through direct dissolution of DSPE-PEG₂₀₀₀ at 1, 2.5 or 5 mM concentrations in isotonic 10 mM HEPES buffer (pH 7.4) by 3min vortexing followed by 10min sonication, as previously described.^{32, 37} Appropriate weight amounts of VIP in 10mM HEPES buffer (pH7.4) were added to preformed SSM and equilibrated for 2 hours at 25°C to form VIP-SSM. Hydrodynamic diameter of the micelles was measured by dynamic light scattering (DLS) using NICOMP 380 particle sizer (Particle Sizing Systems, Santa Barbara, CA). VIP dispersions (free VIP) in isotonic isotonic 10mM HEPES buffer, pH7.4, were prepared by direct dissolution.

Stability of VIP-SSM

Undiluted VIP-SSM and samples after 10- and 100-fold dilution in 10mM HEPES buffer (pH 7.4) were evaluated by size exclusion chromatography (SEC) using a Waters 600 HPLC system (Waters Corporation, Milford, MA). For separation KW-804 Shodex column (8×300 mm, 500A), isocratic flow rate of 1 ml/min and 10mM HEPES buffer (pH 7.4) as a mobile phase were used. VIP detection was achieved by UV at 214 nm, followed by fluorescence, specific to tyrosine residue (excitation 280nm, emission 304nm). The results were expressed as percentage of micelle associated VIP compared to total VIP amount in all chromatographically separated fractions.

VIP-SSM (5mM DSPE-PEG₂₀₀₀, 50μM VIP) and VIP in buffer (50μM) were spiked with ¹²⁵I-VIP (10⁶ cpm). Samples were incubated in absence and presence of 25% and 50% human serum at 37°C and amount of intact VIP was assessed on day 0,1,3,5, and 7. VIP associated with micelles was separated from the free peptide using a Bio-Spin P-30 column, followed by separation of the intact VIP from the micelle fraction by precipitation with 10% trichloroacetic acid (TCA).³⁶ The percent of the intact VIP associated with SSM at each time point was calculated as: [(cpm¹²⁵I-VIP in micelle fraction after TCA precipitation)/(cpm ¹²⁵I-VIP applied to the column)]*100 (cpm stands for counts per minute). The amount of the intact peptide in free VIP samples was assessed by ¹²⁵I residual radioactivity after TCA precipitation.

Aliquots of VIP-SSM at lipid concentration of 1, 2.5 or 5 mM and fixed VIP concentration of 50 μ M, as well as blank SSM at respective lipid concentrations were flashed with argon, sealed and stored at 5 and 25°C in dark for 28 days. Visual appearance (color, clarity, and presence of precipitate) was monitored throughout the observation period. Turbidity of the samples was evaluated by UV-Vis absorbance at 360nm ($A_{360\text{nm}}$) on day 0, 1, 3, 5, 7, 14 and 28.

Induction and assessment of collagen induced arthritis *in vivo*

All animal studies were carried out in accordance with the UIC Institutional Animal Care Committee guidelines. A well-defined model of CIA was used.^{17, 39} Briefly, bovine type II collagen (CII) was dissolved in 0.05M acetic acid at 4°C overnight, and emulsified with an equal volume of complete Freund's adjuvant. Male 6–10 week-old DBA/1J mice (Jackson Laboratory, Bar Harbor, ME) were subcutaneously injected at the base of the tail with 0.15 ml of the emulsion, containing 200 μ g of CII. On day 21 after the primary immunization mice were boosted intraperitoneally with 200 μ g CII dissolved in phosphate buffered saline (PBS). Mice were examined every other day and monitored for signs of disease using two parameters, paw swelling and clinical arthritis score. Paw swelling was assessed by measuring the thickness of the affected hindpaws with calipers. Clinical arthritis score was assessed by the following system: grade 0 - no swelling; grade 1 - slight swelling and erythema; 2 - pronounced edema; 3 - joint rigidity. Each limb was graded, giving a maximum possible score of 12/animal.

Treatment protocols

CIA mice after secondary CII immunization were randomly assigned into groups of six animals each and treatment was immediately initiated. VIP-SSM and free VIP at various peptide doses, as well as vehicle controls (SSM and HEPES buffer) were administered either intravenously (IV) on day 22 post primary immunization or subcutaneously (SC) on days 22 and 34.

Radiological and histological analysis

Mice were sacrificed by CO₂ asphyxiation on day 58 after primary immunization. Hind paws were fixed with 10% paraformaldehyde and radiographs were taken using a General Electric mammography X-ray machine (UIC Hospital, Department of Radiology). The following parameters were used: 9mA, 26 kV, magnification factor 1.8 and a focal spot size of 0.1mm for enhanced sensitivity and precision.

For histological analysis, hindpaws were collected, fixed in 10% paraformaldehyde, decalcified in 5% formic acid, embedded in paraffin, and microtomically sectioned. Five micrometer tissue sections were stained with hematoxylin/eosin/safranin O.

Pharmacokinetics (PK) and biodistribution (BD) of VIP-SSM and free VIP in mice with CIA

DBA/1J male mice were induced with CIA as described above. Arthritic animals were intravenously injected via the tail vein with either VIP-SSM or free VIP (5.0nmol/animal), spiked with 10⁶cpm ¹²⁵I-VIP. Mice were sacrificed 1,2,5,10,15,30 min and 1,2,24 hours later by CO₂ asphyxiation.

For BD analysis animal organs (lung, kidney, spleen, liver, as well as hind and fore limbs) at each time point were immediately dissected, weighed, washed with saline and placed in tubes (0.5g/organ), followed by homogenization with a tissue homogenizer in PBS on ice. Total protein was precipitated by 10% TCA for 90 min, followed by sample centrifugation at 400g for 15 min. Mean amount of intact VIP in the organs at each time point was measured as a residual ¹²⁵I radioactivity in the protein pellet using a Packard Cobra 5005

gamma counter (PerkinElmer, Waltham, MA). The total VIP tissue exposure was computed as the area under the concentration-time curve (AUC_{0-24h}) for different organs by the trapezoidal rule.

Animal blood was drawn by cardiac puncture at each time point (1, 2, 5, 10, 15, 30 min, and 1, 2, 24 hours) and PK parameters were calculated using residual ¹²⁵I-VIP radioactivity in whole blood. The pooled data from all animals were fit with two- or three- compartment models using an ordinary least squares method implemented with the Solver Add-in in Microsoft Excel (Microsoft Corp., Redmond, WA).

A sum of exponential terms adequately fit the observed concentration-time data

$$C = \sum_{i=1}^n X_i \cdot e^{-\lambda_i t}, \quad (\text{Equation 1})$$

, where C is the blood concentration, t is time, i=1 to n represents the number of compartments and X and λ are constants to be estimated.

The following PK parameters were then calculated: initial distribution half-life ($t_{1/2\alpha}$) = $\ln(2)/\lambda_1$; terminal phase half-life ($t_{1/2\beta}$) = $\ln(2)/\lambda_n$; blood clearance (CL) = Dose (10^6 cpm)/AUC, where AUC was calculated using Eq.2; and mean residence time (MRT) = AUMC/AUC, where area under moment curve (AUMC) was calculated using Eq.3.

$$\text{AUC} = \sum_{i=1}^n \frac{X_i}{\lambda_i} \quad (\text{Equation 2})$$

$$\text{AUMC} = \sum_{i=1}^n \frac{X_i}{\lambda_i^2} \quad (\text{Equation 3})$$

Effects of VIP-SSM on systemic arterial pressure (SAP) in mice with CIA

A tail-cuff blood pressure measuring method with an aid of software provided by the device manufacturer (Kent Scientific Corp., Torrington, CT) was used for this purpose. CIA mice were acclimatized in a heated restrainer for 10min daily for 10 consecutive days. On the day of the experiment, each animal was placed in the restrainer for 10 min followed SAP baseline recording. Then, the tail was anesthetized with 10% lidocaine jelly to minimize tail flinching and VIP-SSM and free SSM at various peptide doses were intravenously injected. SAP of the animals was monitored for up to two hours post drug administration.

Detection of circulating levels of RA markers in mouse serum

Concentration of pro-inflammatory (TNF- α , IL-1 β) and anti-inflammatory (IL-4, IL-10) agents, as well matrix metalloproteinases (MMP-2, MMP-9) in mouse serum were detected by ELISA, according to the manufacturer instructions of the respective kits. Serum was separated from blood drawn on day 58 after primary immunization from CIA mice treated with various regiments of VIP-SSM and free VIP as well as vehicle controls (SSM and buffer) by centrifugation at 1200g for 15 min at 4°C. Prior the analysis, isolated serum was diluted 50-fold with an assay buffer, supplied with the respective ELISA kit.

Statistical analysis

Differences between the groups were compared by two-tailed Student's t-test. For multiple (three and more) groups one-way analysis of variance (ANOVA) was used. The Mann-Whitney *U*-test to compare nonparametric data for statistical significance was applied on all clinical results and ELISA experiments. Differences were considered significant when $p < 0.05$.

RESULTS

Characterization and optimization of VIP-SSM

In the initial part of this study, VIP-SSM formulation was optimized and characterized *in vitro*. For this purpose, VIP-SSM at various peptide/lipid compositions was evaluated for its dilution, serum, and short-term stability profiles. All analyzed VIP-SSM formulations had monomodal particle size distribution with mean particle size of 15.3 ± 2.0 nm determined by DLS (intensity-weighted Nicomp distribution), which were not significantly different from the blank SSM (15.1 ± 1.8 nm), corresponding to our reported size data.³⁷ Additional information on the particle size distribution and peptide/lipid association of VIP-SSM is provided in the supplementary information (Supplementary Figure S1).

Effect of dilution on VIP-SSM

One of the important characteristics of any micellar intravenous dosage form is to withstand dilution in a bloodstream on administration. Anticipated dilution of VIP-SSM on intravenous injection to mice was approximated to be nearby 100-fold. VIP-SSM with various lipid content (1, 2.5, and 5 mM) and fixed VIP concentration of $50 \mu\text{M}$ was diluted with isotonic HEPES buffer at 10- and 100-fold, followed by SEC analysis. A significant decrease in micelle associated VIP for 1mM lipid was observed with 10-fold dilution, and at 100- fold dilution for both 1 and 2.5 mM lipid formulations (Figure 1). On the other hand, dilution up to 100-fold of VIP-SSM at 5 mM lipid did not significantly decrease amount of micelle associated VIP in comparison with undiluted samples (Figure 1), suggesting appropriate ratio of active ingredient to excipient for *in vivo* application.

Serum stability of VIP-SSM

Rapid degradation of VIP on systemic administration²¹ hinders, in part, its clinical use. Accordingly, stability of VIP in absence and presence (25% and 50%) of human serum was assessed in simulated physiological conditions (37°C , pH7.4). Within first 24 hours of incubation more than 70% of VIP dispersed in HEPES buffer (free VIP) was degraded, followed by near complete peptide degradation by day 7 in all tested media (Figure 2a). Whereby, association of VIP with SSM conferred significant *in vitro* stability to the peptide. During first five days of VIP-SSM incubation degradation of VIP was non-significant (Figure 2b). By day 7 near 90% of the peptide remained intact in absence of serum, almost matching the values of 86% for VIP-SSM samples incubated in the presence of serum (Figure 2b). Collectively, our findings provide evidence that VIP association with SSM precludes hydrolytic or/and enzymatic peptide degradation in presence and absence of serum components. These data also corroborate our previous findings on protection of SSM associated peptides against enzymatic decomposition.^{29,31}

Short-term Stability of VIP-SSM

VIP-SSM (1, 2.5 and 5 mM DSPE-PEG₂₀₀₀, $50 \mu\text{M}$ VIP) in a liquid state was evaluated for stability at different temperatures, suitable for storage of an injectable dosage forms, specifically refrigeration (5°C) and room temperature (25°C). Visual appearance (color, clarity, and precipitation) and turbidity ($A_{360\text{nm}}$) of the samples were evaluated. VIP-SSM

dispersions stored at 5°C maintained clarity and transparency within the observation period (28 days of storage). Refrigerated VIP-SSM samples did not show any significant turbidity increase for up to 28 days (Figure 3a), confirming the visual observations. On the other hand, a concentration dependent increase in cloudiness, apparent precipitation, and turbidity (Figures 3b) was observed for samples kept at room temperature (25°C) beyond 7 days of storage. This increase in the particulate formation was primarily attributed to the degradation of the phospholipid itself as evidenced by the notable turbidity ($A_{360\text{nm}}$) enhancement for blank SSM stored at 25°C in comparison with refrigerated at 5°C SSM (Figures 3c, d).

Based on the characterization data obtained from this initial part of the study, composition of the optimized VIP-SSM formulation was found to be 5.0mM DSPE-PEG₂₀₀₀ with 50 μ M VIP. Therapeutic efficacy of the optimized SSM-VIP against experimental arthritis was further evaluated in the second part of the study.

In vivo studies on VIP-SSM

Anti-inflammatory and immunomodulatory *in vivo* efficacy of VIP against experimental arthritis was previously studied by several research groups.^{17,18} However, short peptide half-life²¹ and potential side-effects^{40, 41} impede clinical application of VIP. Moreover, to achieve substantial results repetitive administration and relatively high peptide dose were required. Based on our previous work on peptide-SSM nanomedicines^{28,29,31,42} and encouraging *in vitro* data described above, we hypothesized that the potential drawbacks of the native free peptide administration could be overcome by VIP association with SSM. As a result of expected prolonged peptide circulation and specific delivery of the entrapped cargo to the inflamed tissue by the nanosized carrier SSM, we anticipated that lower dose of VIP in SSM will be required to achieve similar therapeutic effect in comparison with free peptide. Accordingly, this hypothesis was tested on a well-defined model of RA, collagen induced arthritis.^{17,39}

Efficacy studies on VIP-SSM against Collagen Induced Arthritis

In the first part of the efficacy studies, intravenous (IV) therapy was assessed. DBA/1J mice induced with CIA were injected at the disease onset (day 22 post primary collagen immunization) with VIP-SSM or free VIP. The peptide dosing was based on the previously reported studies using free VIP to treat CIA in mice.^{17,18} As the starting point the lowest dose shown to be effective in those studies, 5.0 nmol VIP per animal, was selected followed by 5- and 10-fold lower dosing regimens corresponding to 1.0 and 0.5 nmol/animal respectively.

Treatment with a single low IV dose of VIP-SSM (0.5 nmol/animal) had similar anti RA effect as a 10-fold higher dose of free VIP (5.0nmol/animal) evaluated by paw thickness and clinical arthritis score (Figures 4a and 4b). There was some trend of improvement in efficacy of VIP-SSM with elevation of the peptide dose; however, this therapeutic dose response for VIP-SSM was not significant (Figures 4c and 4d). All tested doses of VIP-SSM administered on day 22 post primary immunization as a single injection were sufficient to sustain protection from CIA during remaining duration of the study. At the same time, it is important to note that free VIP demonstrated a significant reduction in clinical signs of arthritis only at 5.0nmol dose, whereas 0.5 and 1.0 did not show any therapeutic efficacy (Figure 4c, d). There was no significant difference in paw thickness and clinical arthritis score among the control groups injected with empty carrier or buffer (Figure 4a, b, c, d).

At the conclusion of the study (day 58), histopathological and radiographic analyses of joints of mice treated with VIP-SSM, showed complete abrogation of CIA-characteristic chronic inflammation of synovial tissue (infiltration of mononuclear cells into the joint

cavity and synovial hyperplasia), pannus formation, cartilage destruction, bone erosion, and joint deformity in comparison to control treated animals (Figure 4e and 4f).

Furthermore, we also tested the efficacy of VIP-SSM by subcutaneous (SC) route, since SC administration is less invasive and potentially more acceptable for patients due to self-administration. In our preliminary studies, a single 5.0nmol/animal dose of VIP-SSM was used. However, the signs of disease recurrence were observed in approximately 3 weeks after the treatment cessation. Therefore, the treatment protocol was altered to include two separate injections on both day 22 and 34 after primary CII immunization. Animals treated with VIP-SSM by this regiment demonstrated similar improvement in paw thickness and clinical arthritis score (Figures 5a and 5b) to a single low-dose intravenous VIP-SSM (0.5nmol/animal). However, therapeutic effect of subcutaneously administered free VIP was modest. The disease was only suppressed for about a week after cessation of free VIP treatment (Figure 5) between days 34 and 40, after which paw swelling and clinical arthritis score started to climb up, reaching levels of control-treated animals at the end of the observation period (day 58).

In a separate study, we also tested efficacy of VIP-SSM against established CIA. Mice were intravenously injected with VIP-SSM and free VIP on day 34 post primary immunization, when the clinical score reached value of 4 or above. Treatment of CIA mice with established RA offered similar protection against the disease (Supplementary information Figure S2), suggesting that the single IV dose of low-dose VIP-SSM is sufficient to ameliorate the pathologic signs of progressed arthritis.

VIP toxicity studies

Systemic administration of free VIP has been markedly limited due to its native vasodilatory property that provokes hypotension.^{40,41,43} Administration of VIP, but not VIP-SSM elicited a significant rapid dose-dependent drop in systemic arterial blood pressure (SAP) of arthritic mice (Figure 6a and 6b). SAP of arthritic mice injected with sub-therapeutic dose of free VIP (0.5nmol) rapidly fell to about 25% beneath baseline level within first 3 min post administration. Whereas, SAP of mice injected with 5.0nmol free VIP dropped to rampant level of near 50% below baseline. The full recovery of SAP to the norm after single free VIP injection required approximately one hour, regardless of dose. VIP-SSM at both peptide doses tested (0.5 and 5.0nmol) completely abrogated hypotensive side-effect observed on free VIP administration (Figure 6a, b).

These observations give evidence that the optimized formulation of VIP-SSM not only therapeutically more effective, but also limits the systemic toxicities associated with aqueous VIP administration, in particular, hypotension.

Pharmacokinetics and biodistribution of VIP-SSM

Various pharmacokinetic parameters and the biodistribution of VIP-SSM were compared with those of free VIP after a single bolus IV injection to CIA mice. A characteristic two-compartment profile described the decay of VIP in whole blood over time, while three-compartment model was required to adequately characterize VIP-SSM profile. Residual blood radioactivity over time data suggested that both free VIP and VIP-SSM were rapidly distributed to tissues following injection in mice with CIA, with initial distribution half-lives calculated to be less than 2 min. Obtained PK parameters for free VIP were similar to those reported prior for healthy rodents.^{36,44} The terminal phase half-lives and mean residence time (MRT) of free VIP in comparison with VIP-SSM were markedly different (Table 1). Aqueous VIP was rapidly cleared from the body with negligible concentrations detected at 24 h while substantive concentrations of VIP-SSM were measured after the same period,

reflecting approximately 150-fold slower clearance of VIP-SSM in comparison with free VIP (Table 1).

Further, biodistribution data from various organs showed that the majority of residual radioactivity for free VIP accumulated in the liver and kidney, whereas VIP-SSM accumulated in these RES organs as well as in inflamed joints of CIA mice (Figure 7). Accumulation with time of both VIP-SSM and aqueous VIP in various organs of CIA mice is depicted in Supplementary figure S2. Moreover, VIP-SSM quickly saturated the joints of arthritic mice within minutes (<10 min) with ~3% of injected dose with sustained levels of detectable VIP radioactivity at least up to 24h as determined end of the study (Supplementary Figure S2). As a result, a 13-fold higher total accumulation of VIP-SSM was found in joints of CIA mice in comparison with free VIP (Figure 7), while at the end of the study (24h) the difference was 23-fold (Supplementary Figure S3).

VIP-SSM inflammatory response studies in CIA

Wide array of cytokines and chemokines is involved in progression of inflammation and development of RA.^{1,45} The most relevant production site of these agents in arthritis is the synovial milieu of RA-affected joints. Many of these inflammatory modulators are excreted to the blood circulation and their levels correspond to the RA progression and the treatment response.⁴⁶

Accordingly, the circulating levels of several disease markers were determined in serum of CIA mice treated with VIP-SSM in comparison with free VIP and controls (buffer, blank SSM). CIA-induced expression of pro-inflammatory factors TNF- α and IL-1 β were inhibited following all treatment regimens of VIP-SSM (Figures 8a and 8b). At the same time, circulating levels of the anti-inflammatory cytokines IL-10 and IL-4 were significantly increased in comparison with control even at 0.5nmol of VIP-SSM (Figures 8c and 8d). In comparison, only the high dose (5.0 nmol) intravenous aqueous VIP elucidated similar results to VIP-SSM (Figure 8a).

Matrix metalloproteinases (MMPs) play pivotal role in the depletion of proteoglycan and collagen in inflamed joints, leading to cartilage and bone erosion in RA patients.⁴⁷ We therefore assessed whether VIP-SSM influenced circulation levels of MMPs in CIA mice. VIP-SSM treatment at all dose levels and regimens significantly inhibited circulating levels of collagenase MMP-2 and MMP-9 in mice (Figure 8e and 8f).

Together, these results suggest that the treatment with VIP-SSM reduces the inflammatory response by downregulating expression of pro-inflammatory and upregulating expression of anti-inflammatory agents. The inhibitory effect of VIP-SSM on MMP-2 and MMP-9 could be directly related, at least in part, to the VIP-mediated inhibition of cartilage destruction and bone erosion, observed histologically and radiographically.

DISCUSSION

Peptides have gained much attention as bio-inspired therapeutic agents for many years. Advances in biotechnological manufacturing and purification led to new upsurge in the area and rapid development of peptide-based therapeutics for various medical conditions. However, challenges associated with peptide stability and proper delivery stratagems still preclude revelation of the full therapeutic potential of these entities and consequently clinical use.³¹

Vasoactive intestinal peptide was shown to be effective in ameliorating CIA in rodents.^{17,18} However, short circulation half-life²¹ and serious side effects, such as acute

hypotension^{40,43} and potential dysregulation of gastrointestinal system and host defense on chronic peptide administration,⁴⁸ hindered clinical application of VIP. Our strategy exploited versatile properties of SSM nanocarrier in peptide modulation, improvement of pharmacokinetic parameters, and preferential delivery of the entrapped cargo to the site of action. This approach offered therapeutic efficacy of low-dose VIP in treatment of experimental arthritis in a safe and stable manner.

Here, we demonstrated that endowed spontaneous interaction of VIP with SSM conferred significant stability to the peptide. Micelle systems are influenced by dilution due to the dynamics of lipid monomers to micelles equilibrium. In contrast to many conventional surfactant micelles, SSMs are relatively stable against dilution, owing to extremely low critical micelle concentration of DSPE-PEG₂₀₀₀ (~1 μ M).⁴⁹ Even then, to make up for the decreased monomer concentration, SSM breakdown should lead to the release of the entrapped peptide cargo. However, at high lipid concentration the breakage of peptide containing micelles are so small that insignificant peptide release is achieved. For tested VIP-SSM, this concentration was apparently achieved at 5mM lipid or near one peptide molecule per micelle, estimated accordingly to known SSM aggregation number.⁴⁹ Association of VIP with SSM also protects the peptide from enzymatic degradation that was assessed by incubation of VIP-SSM in presence of human serum. This phenomenon could be attributed, at least in part, to peptide shielding in PEG palisade of SSM as well as peptide stabilization in proteolysis-resistant α -helix form.³³ The former notion is in compliance with our previous work on VIP interaction with conventional liposomes that resulted in conformational shift of the peptide toward α -helix and significant stabilization of liposomal VIP in the presence of human serum for up to 7 days.³⁶ Our data also suggest that preferable condition for the short-term storage of VIP-SSM dispersions is refrigeration, allowing multiuse of the liquid dosage form after reconstitution of freeze-dried formulation. For their long-term storage VIP-SSM must be stored as freeze dried form which can be easily achieved without use of any additional cryo- or lyoprotectants.³⁷

Stabilization of VIP by SSM was also observed *in vivo*, reduced systemic clearance and prolonged circulation time of VIP-SSM resulted in 39-fold longer mean residence time in the body compared with free VIP (19.5h versus 0.5h, respectively). Biodistribution data supported pharmacokinetic results since 13-fold higher accumulation of VIP-SSM in the diseases joints was obtained in comparison with free VIP. Thereby, this allowed for ~3% of injected dose to saturate the joints within minutes of administration and maintain sustained levels till the end of the study (24h).

Certainly, the steric hindrance conferred by the PEG molecules grafted on the surface of SSM that evade uptake by the reticuloendothelial system⁵⁰ and enhanced permeability and retention effect in inflammation^{51,52} played an important part in the preferential targeting of nanosized VIP-SSM to the inflamed milieu overexpressing VIP receptors, RA joints. Thus, these distinct features of VIP-SSM lead to a reduced dose of active VIP molecules required to achieve desired biological response in mice with CIA, animal model that resembles many similarities to human RA.³⁹ This study is the first to show, that circulation levels of critical players of the pathogenesis of RA in response to VIP treatment correlated with clinical findings on joint inflammation and deterioration of CIA mice. Even though, dose escalation and double subcutaneous injection of VIP-SSM was required to achieve similar activity to the low-dose intravenous therapy, it is important to note that for human use subcutaneous application may be preferential due to convenience for self-injection. Nevertheless, VIP-SSM therapy against CIA was superior to free VIP regardless of the route of administration. Recently, potentiated anti-inflammatory efficacy of steroids delivered by a different nanocarrier, stealth nano-liposomes, via intravenous and subcutaneous administration in polyarthritis was also reported.⁵³ In another study, we have previously used VIP (at 10-fold

lower dose than here) as a targeting agent to successfully deliver camptothecin in micelles to the inflamed tissues of CIA mice.⁵⁴

Vasodilatory property of VIP was a major challenge addressed in this study. In order to exert its hypotensive side-effects, VIP has to extravasate out of the circulation to reach its target receptors expressed on smooth muscle cells at the abluminal side of vasculature.⁵⁵ To this end, the size of SSM-VIP, ~15nm, prevent the construct to extravasate through intact blood vessel wall abrogating vasorelaxation and furthermore delivering the particles to the sites with 'leaky' vasculature, such as inflamed tissues.

The VIP-SSM manufacturing process, compared to many other nanoconstructs, is straightforward, robust and non-labor consuming. The components of VIP-SSM formulation have been used for humans as ingredients of marketed pharmaceutical products, VIP in Invicorp® and DSPE-PEG₂₀₀₀ in Doxil®. Biocompatibility of VIP-SSM components is one of attractiveness for its substantial clinical use.

We can conclude that single, intravenous low-dose VIP in biocompatible, biodegradable phospholipid micelles (VIP-SSM) has a profound therapeutic effect on CIA without associated hypotensive side-effects of free VIP. We propose, further evaluation of VIP-SSM as an attractive disease modifying nanomedicine for rheumatoid arthritis and other chronic inflammatory and autoimmune disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported, in part, by NIH grants AG024026, HL72323 and CA121797, and by VA Merit Review. This research project was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06RR15482 from the National Center for Research Resources, NIH.

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Figure 1. Dilution effect on VIP-SSM: SSM at 5.0mM lipid conserved micelle associated VIP up to 100-fold dilution in comparison with 1.0 and 2.5mM lipid. Values are mean $\pm$ SD for 4 separate experiments; * $p < 0.05$ in comparison to respective undiluted samples; NS – nonsignificant.

Figure 2. Stability of VIP and VIP-SSM in presence and absence of human serum following incubation at 37°C for 7 days, percentage of remaining intact VIP determined by size-exclusion chromatography: **(a)** free VIP; **(b)** VIP-SSM. Values are mean $\pm$ SD for 4 separate experiments; * $p < 0.05$ versus day 0.

Figure 3.

Stability of VIP-SSM (1.0, 2.5 and 5.0 mM lipid; 50 μ M peptide) and blank SSM (1.0, 2.5 and 5.0 mM lipid) following storage at various temperatures for 28 days assessed by turbidity (A_{360nm}): (a) VIP-SSM at 5°C; (b) VIP-SSM at 25°C; (c) SSM at 5°C; (d) SSM at 25°C; Values are mean $\pm$ SEM for 4 separate experiments; * $p < 0.05$ versus day 0.

Figure 4. SSM-VIP exerts anti-CIA action at lower VIP doses. CIA mice were treated intravenously on day 22 post primary immunization. Progression in (a) paw thickness and (b) clinical arthritis score of CIA mice treated with VIP-SSM (0.5nmol), free VIP (5.0nmol), and vehicle controls (buffer and SSM). ↑ -represents dosing; change in (c) paw thickness and (d) clinical arthritis scores of CIA mice in comparison to baseline at the end of observation period (day 58) injected with various doses of VIP-SSM, free VIP, and vehicle controls (buffer and SSM). Results are expressed as mean $\pm$ SD from 6 mice/group. # $p < 0.05$ versus control treated; * $p < 0.05$ versus respective dose of free VIP treatment. (e) Representative histology joint sections of buffer (left) and 1.0nmol VIP-SSM (right) treated mice on day 58 (the bar represents 500 μ m; P.F.- pannus formation; C.D. – cartilage destruction; B.E. – bone erosion). (f) Representative hindlimb radiographs of buffer (left) and 1.0nmol VIP-SSM (right) treated mice on day 58.

Figure 5.

Efficacy of subcutaneously administered VIP-SSM against CIA. Progression in **(a)** paw thickness and **(b)** clinical arthritis score of CIA mice treated with VIP-SSM (5.0nmol), free VIP (5.0nmol), and controls (buffer and SSM) in two doses on day 22 and 34 post primary immunization. ↑ -represent dosing. Results are expressed as mean $\pm$ SD from 6 mice/group.

Figure 6.

VIP-SSM diminishes hypotensive side-effects of free VIP. Systemic arterial blood pressure (SAP) of CIA mice following administration of single dose of VIP-SSM was within baseline level in comparison with precipitous drop in SAP on free VIP administration: peptide dose (a) 0.5nmol and (b) 5.0nmol. Results are expressed as mean $\pm$ SD from 6 mice/group.

Figure 7. Comparative tissue distribution (AUC_{0-24h}) of VIP in CIA mice following intravenous administration of VIP-SSM and free VIP (5.0nmol/animal spiked with 10^6 cpm of ^{125}I -VIP). Results are expressed as mean $\pm$ SD from 6 mice/group; * $p < 0.05$ in comparison with aqueous VIP in a respective tissue.

Figure 8.

VIP-SSM inhibits inflammatory response in CIA at a greater extent than free VIP. Circulating levels of proinflammatory agents (a) TNF- α and (b) IL-1 β ; anti-inflammatory cytokines (c) IL-4 and (d) IL-10; as well as matrix metalloproteinases (e) MMP-2 and (f) MMP-9 were determined at the end of the study (day 58) in serum of CIA mice treated with various concentrations of free VIP or VIP-SSM administered either intravenously (IV) or subcutaneously (SC) on day 22 (as a single injection) or days 22 and 34 (two separate doses), respectively (as indicated in the figure legend). Results are expressed as mean $\pm$ SD from 6 mice/group. * p <0.05 versus respective control treatment, # p <0.05 versus VIP (0.5nmol, IV), ^ p <0.05 versus VIP (5.0nmol, SC).

Table 1

Pharmacokinetic (PK) parameters calculated following administration of either free VIP or VIP-SSM to CIA mice.

PK Parameter	free VIP	VIP-SSM
Initial distribution half-life ($t_{1/2,\alpha}$)	1.2 min	0.9 min
Terminal phase half-life ($t_{1/2,\beta}$)	22.6 min	10.9 h
MRT	27.5 min	19.4 h
CL	5.9 ml/min	0.04 ml/min

Data are mean values from pooled analyses. VIP-SSM and free VIP were administered as a single bolus intravenous injection at a peptide dose of 5.0nmol/animal, spiked with radioactively labeled peptide ^{125}I -VIP at 10^6 cpm/animal.