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COMMUNICATION

Immunomodulatory Vasoactive Intestinal Peptide Amphiphile Micelles

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Two different vasoactive intestinal peptide (VIP) amphiphiles have been formulated which readily form micelles of varying shapes. Interestingly, VIP micelle structure has been found to directly correlate to anti-inflammatory behavior providing evidence that these biomaterials can serve as a promising new therapeutic modality.

Main Text

Introduction: Vasoactive intestinal peptide (VIP) is a 28-amino acid neuropeptide that has distinct anti-inflammatory effects including downregulating TNF-α production by activated antigen presenting cells (APCs), specifically macrophages (MØs) and dendritic cells (DCs).[1-3] It has also been shown to induce DCs to secrete CCL22 which recruit regulatory T cells (T_{reg}s) that can facilitate localized tolerance.[1-3] These immunomodulatory effects have led to the extensive research of VIP as a treatment for a variety of autoimmune diseases such as rheumatoid arthritis,[4, 5] multiple sclerosis,[6, 7] and type 1 diabetes.[8, 9] Though exciting, VIP-based therapeutics possess drawbacks similar to other peptide-based therapies including a short-half life and minimal local retention when delivered *in vivo*. Thus, designing an appropriate delivery vehicle is crucial for optimizing the therapeutic efficacy of VIP.

Peptide amphiphiles (PAs) are a unique biomaterial comprised of therapeutic peptide(s) covalently conjugated with hydrophobic lipid(s).[10-16] These di-block materials readily undergo self-assembly in water to form peptide amphiphile micelles (PAMs) due to hydrophobically-driven self-assembly. PAMs have been shown to possess several advantageous properties over peptides alone including increasing local concentration,[17, 18] preventing dissemination,[19] and enhancing cellular interactions.[20] These desirable characteristics have led to PAMs being studied as therapeutic systems for a variety of biomedical applications including regenerative medicine,[21, 22] cancer therapy,[23] and vaccination.[24, 25] In this study, VIP amphiphiles (VIPAs) were created to investigate their capacity to form micelles (VIPAMs) and potentiate the bioactivity of VIP. Physical and biological characterization experiments revealed unique properties for each formulation suggesting VIPAMs hold tremendous potential as a new treatment modality.

VIPA design and physical characterization: Based on our recent research,[26, 27] two VIPA chemistries were produced. The first VIPA was synthesized by directly conjugating palmitic acid (Palm) to the N-terminus of VIP to form Palm-VIP (pVIPA – **Figure 1a and Movie S1**). The second VIPA included a zwitterion-like peptide region between Palm and VIP yielding PalmK-$(EK)_4$-VIP (pzVIPA – **Figure 1b and Movie S2**). Micellization of each VIPA was characterized using a critical micelle (CMC) assay and negative-stain aided transmission electron microscopy (TEM). pVIPA was found to have a very low CMC (*i.e.* 0.08 μM) whereas pzVIPA possesses a CMC two orders of magnitude greater (*i.e.* 9.3 μM). While the addition of the hydrophilic block may have been expected to maintain or decrease the CMC, peptide folding to orient the most hydrophilic section externally can induce bending in the PA that may prevent straightforward micellar packing. This phenomenon has been previously observed by other researchers,[28] and though it raises the CMC significantly, 9.3 μM is still likely low enough to be within the VIP therapeutic window.[21] Interestingly, the two VIPAs yielded different micellar architectures with pVIPA and pzVIPA assembling into cylindrical and braided micelles, respectively. These results align with our previously published work showing that diblock PAs like pVIPA commonly form cylindrical micelles[26] and triblock PAs with the same chemical orientation as pzVIPA self-assemble into braided micelles.[27] The cylindrical micelles were found to be several hundred nanometers to a micron in length whereas the braided micelles were about an order of magnitude greater in length. This increased length is likely due

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to the intermicellar electrostatic complexation we have previously described for similar triblock PAs.[27] Generally, particles in this size range have been found to be sterically hindered from interstitial transport[29-31] facilitating their enhanced injection site retention and making them promising candidates for prolonged drug delivery applications. This is further supported by our previous findings that braided PAMs possess limited cell uptake and lymph node drainage capacity making them well suited for sustained, localized VIP delivery.[32] The secondary structure of the three different VIP formulations was characterized by circular dichroism (CD, **Figure S1**). Similar to previously reported observations,[33-35] palmitic acid conjugation to VIP increased peptide β-sheet content. The addition of a zwitterion-like region (*i.e.* $(EK)_4$) increased overall α-helical content which is an expected phenomenon as oligoglutamyllysine is known to possess this secondary structure.[36]

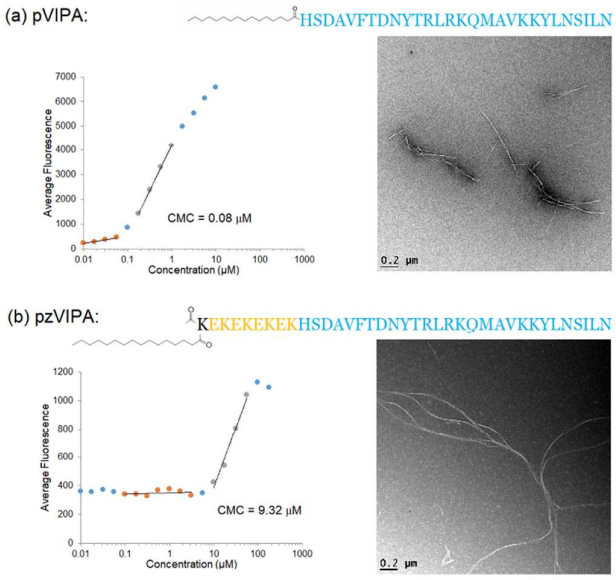

Figure 1. Chemical structure and physical characterization of different VIPAs. At concentrations above their respective CMC values, (a) pVIPA and (b) pzVIPA formed cylindrical micelles and braided micelles, respectively.

VIPAM anti-inflammatory effects: Tumor necrosis factor alpha (TNF-α) is a monocyte-derived cytokine that plays a significant role in the inflammatory response. TNF-α is produced by MØs and DCs that are activated during infection,[37] commonly due to the cell-based identification of pathogen associated molecule patterns, most notably lipopolysaccharide (LPS) found in the cell wall of gram-negative bacteria.[38] Excessive TNF-α production has been shown to cause tissue injury, fever, atherosclerosis, and even death.[39, 40] Unlike activated MØs which accumulate at the site of inflammation, activated DCs tend to migrate to nearby lymph nodes where they activate naïve T helper cells. Activated effector T cells will migrate back to the inflammation site where they will recruit natural killer cells and additional MØs which further exacerbate the inflammatory response. The B7 ligand CD86 present on activated DCs plays an important role in this cascade acting as a co-stimulatory signal for T cell activation. A lack of co-stimulatory signaling often leads to T cell anergy.[41] Conversely, the presence of CD86 on DCs without corresponding MHC II antigen presentation plays a role in T_{reg} induction.[42]

While the capacity to trigger a pro-inflammatory adaptive response is crucial for the host to clear unwanted pathogens, it is also responsible for transplant rejection and autoimmune-mediated tissue damage.[37, 43] One strategy to retard this inflammation loop is to limit TNF-α secretion from activated APCs and CD86 surface presentation on activated DCs. The anti-inflammatory effect of VIPAMs were explored by incubating MØs and DCs with LPS and different VIP materials at low (*i.e.* 1 μM) or high (20 μM) concentrations (**Figure 2**). It was discovered that while VIP alone can modestly reduce TNF-α secretion and CD86 expression, this effect can be modulated through micellar delivery where chemical structure and micellar shape play a crucial role in bioactivity. pVIPA was unable to enhance the TNF-α suppressive effects of VIP in activated MØs (**Figure 2a**) and completely nullified VIP effects on activated DC TNF-α secretion (**Figure 2b**). The only statistically significant anti-inflammatory effect for pVIPA over VIP was found in DC CD86 expression at the high concentration where it was actually enhanced (**Figure 2c**). In contrast, pzVIPA nearly completely abrogated TNF-α secretion in activated MØs (**Figure 2a**), maintained VIP-based TNF-α secretion in activated DCs (**Figure 2b**), and significantly limited CD86 surface expression on activated DCs (**Figure 2c**). Interestingly, these enhancement effects were only observed at the high concentration and not at the low concentration. With a CMC of 9.3 μM (**Figure 1a**), pzVIPA would likely exist as single biomolecules at the low dose (1 μM) and within braided micelles at the high dose (20 μM). In contrast, pVIPA would be confined in cylindrical micelles at both concentrations due to its very low CMC (0.08 μM, **Figure 1b**). To provide further evidence that the observed bioactivity potentiation is a function of certain micelle structures and not the presence of lipid, anti-inflammatory experiments were conducted with Palm alone (**Figure S2**) for which no TNF-α nor CD86 suppression were measured. Taken together, these results indicate that braided VIPAMs possess considerable intrinsic anti-inflammatory properties.

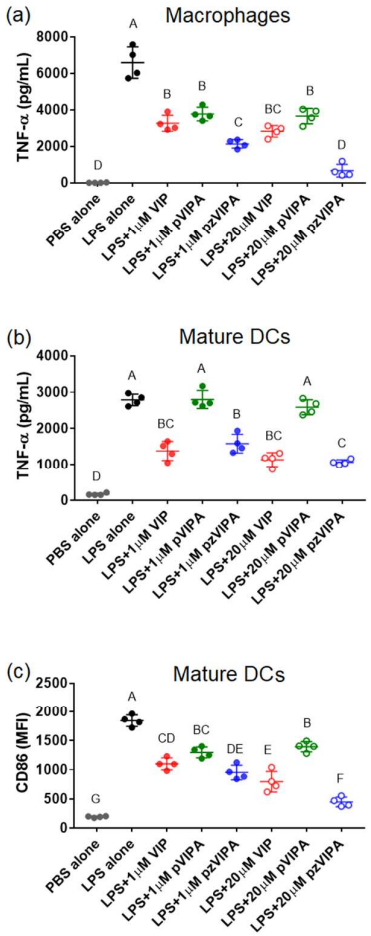

Figure 2. Anti-inflammatory effects of different VIP formulations. TNF-α secretion from MØs (a) or DCs (b) as well as CD86 expression from DCs (c) were evaluated. LPS greatly increased each of these inflammatory correlates which were diminished to variable extents due to the presence of different concentrations and presentations of VIP. Within a graph, groups that possess different letters have statistically significant differences in mean ($p \leq 0.05$) whereas those that possess the same letter are similar ($p > 0.05$).

VIPAM T_{reg} recruitment and induction potential: T_{reg}s are a unique type of suppressor T cell that facilitates peripheral immunological tolerance. Increasing the presence and development of T_{reg}s at the effector site of autoimmunity or inflammation has been suggested as a potential treatment for immune-related disorders or transplant rejection. T_{reg} recruitment to the desirable tissue site can be guided by the presence of a gradient of the chemokine CCL22 (MDC).[41, 44] Previous research suggests that certain concentrations of and incubation times with VIP can induce DCs to produce CCL22 making it a desirable upstream bioactive molecule for T_{reg} recruitment.[45, 46] Thus, we evaluated CCL22 production from DCs treated with different VIP formulations.

Previous results indicate that VIP peptide alone induces significant CCL22 production after 48 hours of incubation.[45] While promising, prior research has shown that the more immediate presence of T_{reg}s is necessary to prevent or treat autoimmune disease and transplant rejection.[47, 48] Our results revealed that while VIP peptide was unable to induce DC CCL22 production at 24 hours, some VIPA formulations were able to provoke appreciable CCL22 increases at this early time point (**Figure 3**). Specifically, the data indicate that high concentration pVIPA and pzVIPA induced greater CCL22 production from immature DCs than those given no stimulus (**Figure 3a**). Interestingly, only high concentration pVIPA enhanced CCL22 production from mature DCs when compared to the LPS-stimulated mature DC control (**Figure 3b**). Similar to the anti-inflammatory studies, lipid presence was found to not be the driving force behind CCL22 induction (**Figure S3**). These differences indicate that pVIPA possesses considerably more intrinsic T_{reg} recruitment potential than VIP or pzVIPA. As VIP-mediated CCL22 induction has a very restricted therapeutic window with regards to both dose and incubation time,[45, 46] future studies are needed to complement this initial result.

Followed by T_{reg} recruitment, the maintenance and expansion of those migrated T_{reg}s are essential for maintaining long term homeostasis.[49] CD86 ligand presented by DCs is an important molecule that has been shown to induce T_{reg} survival and expansion in peripheral tissue, especially in the absence of corresponding MHC II-presented antigen.[42] Therefore, the enhanced expression of CD86 is a potential key factor that affects downstream immunoregulatory functions of CCL22-recruited T_{reg}s. It was discovered that high concentration pVIPA induced the highest CD86 expression of VIP treated groups or lipid control groups for both mature DCs (**Figure 2a**) and immature DCs (**Figure 3c and Figure S4**). The CCL22 and CD86 data suggest that cylindrical VIPAMs possess potential immunoregulatory properties.

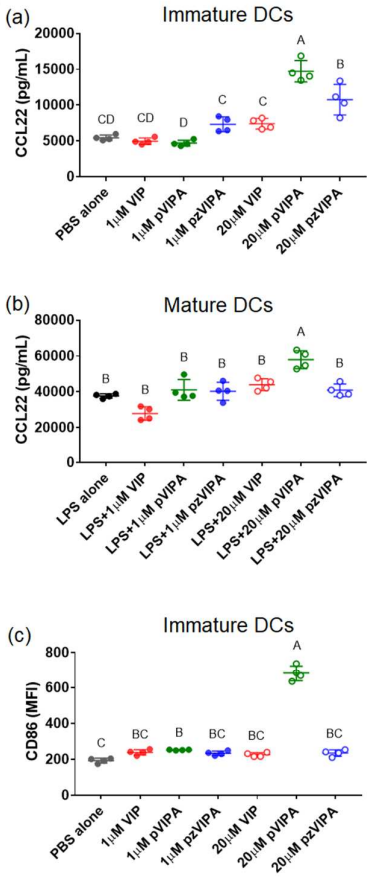

Figure 3. Immunoregulatory effects of different VIP formulations. The secretion of CCL22 from immature DCs (a) and mature DCs (b) as well as the CD86 expression on DCs (c) was evaluated. The production of T_{reg} recruiting CCL22 was enhanced for some VIPAM formulations. pVIPA significantly increased CD86 expression on immature DCs while the other two VIP formulations did not enhance CD86 expression. Within a graph, groups that possess different letters have statistically significant differences in mean ($p \leq 0.05$) whereas those that possess the same letter are similar ($p > 0.05$).

VIPAM Structure-Bioactivity Relationships: Interestingly, VIP is known to modulate TNF-a, CD86, and CCL22 expression through the same receptor (*i.e.* VPAC1),[45, 50, 51] indicating that formulation chemistry and structure is very directly impacting peptide bioactivity. In specific, VIP/VPAC interactions are known to be dependent on a number of factors including VIP concentration, amino acid availability, and conformation.[2, 52, 53] One of the major differences with peptide amphiphiles compared to peptides is their capacity to enhance peptide-cell interactions due to their lipid content.[20, 54-56] Therefore, both pVIPA and pzVIPA are expected to yield greater VIP concentrations at the cell surface. Additionally, the N-terminal amino acid of VIP (*i.e.* histidine) is known to play an important role in VIP/VPAC binding affinity.[53] With the N-terminal histidine on pVIPA being directly lipidated, it is likely to be closer to the membrane and more rigid than pzVIPA which possesses a somewhat flexible linker (*i.e.* $(KE)_4$) between the lipid and VIP. Previous studies have demonstrated VIP α-helicity enhances peptide association with VPAC.[52, 57] Interestingly, CD analysis revealed that pzVIPA had more abundant α-helical conformation than both VIP and pVIPA (**Figure S1**). These factors may impact interactions between pzVIPA and VPAC leading to the enhanced TNF-α and CD86 suppression activity observed (**Figure 2**).

Although both pVIPA and pzVIPA enhanced CCL22 induction from immature DCs (**Figure 3a**), the magnitude of this response was significantly different. DC CCL22 induction is related to different factors including VIP/VPAC engagement and DC activation state.[45] pzVIPA is likely engaging VPAC, but without activating the DCs as no increase in CD86 cell surface expression was detected (**Figure 3c**). In contrast, high concentration pVIPA significantly increased CD86 expression in immature DCs (**Figure 3c**) without altering other stimulatory markers like CD40 expression and TNF-α production (*data not shown*) providing evidence of a semi-mature DC state similar to previous work exploring VIP.[50, 58] This VIP-stimulated DC phenotype was found to directly correspond to more elevated levels of CCL22 production.[45] An across the board increase in CCL22 production for mature DCs (**Figure 3b**) is unsurprising since the production of this chemokine has been shown to be enhanced by LPS stimulation.[59] The further increased CCL22 production by the exposure of mature DCs to high concentration pVIPA may be directly tied to the TNF-α results observed. In specific, prior research has shown that the presence of TNF-α can potentiate the cytokine-inducing capacity of VIP.[2] For LPS-stimulated DCs, pVIPA does not downregulate TNF-α production (**Figure 2b**) though moderates CD86 expression (**Figure 2c**) similar to longer VIP exposure has been previously shown to do.[45] Together these effects provide a strong foundational explanation for why these interesting CCL22 results were detected.

Conclusion: The results shown provide significant evidence that VIP amphiphile chemistry has a profound impact on micelle shape and bioactivity. Though pVIPA and pzVIPA both readily form micelles within the established VIP therapeutic window, each facilitates the formation of a different micellar shape (*i.e.* cylinders or braids). Interestingly, the two VIPAs induced quite different immunomodulatory effects with pzVIPAM braids suppressing the pro-inflammatory behavior of mature MØs and DCs and pVIPAM cylinders stimulating significant CCL22 production from both immature and mature DCs. These data indicate a significant relationship exists between micelle shape and bioactivity. The exciting initial results will be expanded upon through the creation of a pool of different VIPA formulations whose unique micellar nanoarchitectures and bioactivity will be explored. Also, more biochemical experiments will be conducted to better understand the mechanism underpinning the observed cell behaviors. These future efforts will allow for the identification of unique structure-function relationship whose underlying design rules will inform a micellar "tool box" that can be

leveraged for the treatment of a variety of immune-mediated diseases.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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