Anti- Infective Peptides to Enhance the Host Innate Response: Design, Development and Delivery

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Abstract: Background: Inducible Nitric Oxide Synthase (iNOS or NOS2) produces Nitric Oxide (NO) and related reactive nitrogen species, which are critical effectors of the host innate response and play key roles in the intracellular killing of bacterial and parasitic pathogens. The SPRY domain-containing SOCS box proteins SPSB1 and SPSB2 are key physiological regulators of this important enzyme. Disrupting the endogenous SPSB-iNOS interaction should prolong the intracellular lifetime of iNOS and enhance the production of NO, and therefore be beneficial in treating chronic and persistent infections such as tuberculosis. By using structure-based design, potent peptide inhibitors of this interaction have been developed.

Conclusion: Inhibitors of the SPSB-iNOS interaction have therapeutic potential as a novel class of anti-infective agents. Various strategies are being pursued to target these peptide inhibitors to macrophages and deliver them to the cytoplasm of these cells. It will then be possible to assess the efficacy of such inhibitors in boosting the capacity of macrophages to destroy infectious pathogens.

Keywords: Nitric oxide, peptide, structure, protein-protein interaction, pathogen, targeting, macrophage, delivery

1. INTRODUCTION

Antibiotic resistance has been recognized as one of the major challenges facing the 21st century health sector [1-4], with fears expressed that we may soon run out of useful antibiotics for the treatment of infections [5]. As a consequence, there is a pressing need to develop new drugs that combat bacterial disease in ways that are not susceptible to current bacterial resistance mechanisms [6, 7]. This is especially important in combating multidrug-resistant and extensively drug-resistant strains of Mycobacterium tuberculosis [8, 9]. This mini-review describes an approach that addresses this requirement by targeting the host innate immune response to infection rather than the infectious organism itself. As the most potent compound is a peptide and the target is intracellular, this creates the dual challenges of directing the peptide to the relevant cell type (in this case macrophages) and delivering it to the cell cytoplasm.

I begin by outlining the molecular basis of the interaction being targeted, then describe the peptide inhibitors that have been developed, and finally the approaches being applied to deliver these peptides.

2. SPSB-iNOS INTERACTION

2.1. SPSB Proteins

The SPRY domain-containing SOCS box protein 2 (SPSB2) is one of four proteins (SPSB1 to -4) consisting of a C-terminal SOCS box motif [10] and a protein interaction domain known as the SPRY domain [11]. The SPSB proteins function as components of E3 ubiquitin ligases via the C-terminal SOCS box, with the SPRY domain determining the substrates for ubiquitination [12]. SPSB1, -2 and -4, but not SPSB3, interact with prostate apoptosis response protein-4 (Par-4) [13], which is up-regulated in prostate cancer cells undergoing apoptosis [14]. The Drosophila homologue of the SPSB proteins, GUSTAVUS, interacts with the DEAD-box RNA helicase VASA [15].

2.2. SPSB1, 2 & 4 Bind to iNOS via its Conserved N-terminus

The pentapeptide sequences DINNN in Drosophila VASA and ELNNN in human Par-4 mediate their interaction with GUSTAVUS, and SPSB1 & 2, respectively [16]. However, this sequence motif is not present in mouse or human VASA, or in mouse Par-4, suggesting that neither Par-4 nor VASA is the key physiological target of the mammalian SPSB proteins. We therefore sought other possible targets for SPSB proteins. One likely candidate was the inducible form of Nitric Oxide Synthase (iNOS), which contains a highly conserved DINNN sequence in its intrinsically disordered N-terminus [17].

We showed using Isothermal Titration Calorimetry (ITC) that a peptide corresponding to residues 19-31 of iNOS bound to SPSB2 with high affinity (Kd, 13 nM) [17]. Mutation of Asp23, Asn25 or Asn27 (DINNN) to Ala dramatically reduced the affinity [17]. Conserved residues flanking the DINNN sequence (Lys22, Val28 and Lys30) made only minor contributions to binding.
High-resolution NMR was used to further characterize the SPSB2-iNOS peptide interaction. Titration of the unlabelled iNOS N-terminal peptide into $^{15}$N-labelled SPSB$_{12}$ caused a gradual disappearance of a subset of free SPSB2 cross-peaks and the simultaneous appearance of a ‘bound’ subset of cross-peaks in $[^1H,^{15}N]$-HSQC spectra. The residues that exhibited chemical shift perturbations lay on a continuous surface on SPSB2 in the vicinity of Tyr120, Val206, and Trp207, representing the iNOS peptide-binding site [17].

2.3. High-resolution Crystal Structures For SPSB-Peptide Complexes

Subsequently, crystal structures were determined for human SPSB1 (the prefix h refers to human in the subsequent text, and m murine) in complex with hPar-4$_{67-81}$ and VASA$_{184-203}$, as well as the hSPSB2/VASA complex, apo-hSPSB4 [18] and apo-mSPSB2 [19]. The structure of hSPSB1 bound to hPar-4$_{67-81}$ enabled direct comparison of the hPar-4 and VASA binding modes in hSPSB1 (Figure 1A). Residues Asn67-Pro74 from hPar-4 were well defined, corresponding to the minimal binding motif determined by NMR chemical shift changes. Further electron density was not present to build the C-terminal residues (75-81, GGA-PAAP) suggesting that, as for VASA, the N-terminal site is sufficient for the high-affinity interaction. The hPar-4 and VASA peptides adopt similar conformations, with the conserved NNN motifs forming a total of six conserved hydrogen bonds (Figure 1B & C). An additional main chain hydrogen bond is formed between Asn70 of hPar-4 and the Gly218 amide of hSPSB1; this position is deeply buried by the close packing on either side of Tyr129 and Trp217 from hSPSB1.

Figure 1. Peptide binding to hSPSB1. (A) Overlay of hSPSB1 structures in complex with VASA (dark green) and hPar-4 (light green). Surface of hSPSB1 is coloured by electrostatic potential. (B) Side-chain H-bonds formed by the central hPar-4 NNN motif. Similar bonds are formed in the VASA complex. (C) Intramolecular H-bonding in hPar-4 (L) and VASA (R). VASA is stabilized by an additional main-chain H-bond (D184 - N188). Reproduced with permission.
2.4. SPSB1 and SPSB2 Coordinate to Regulate iNOS Expression

Extensive biochemical analyses demonstrated that iNOS and SPSB2 could be found in an endogenous complex in Bone Marrow-Derived Macrophages (BMDM) and that SPSB1, SPSB2, SPSB4, but not SPSB3, interacted with iNOS [17]. To determine whether the kinetics or magnitude of iNOS expression were altered in the absence of SPSB2, BMDM from SPSB2-deficient mice (Spsb2−/−) or wild-type littermates were stimulated with Lipopolysaccharide (LPS)/Interferon (IFN)-γ for various times, then lysed and iNOS expression detected by Western blot. Although the initial kinetics of iNOS induction appeared to be the same in both wild-type and Spsb2−/− BMDM, there was a modest increase in iNOS protein in Spsb2−/−[17]. A greater difference in iNOS expression between wild-type and Spsb2−/− BMDM was evident after the stimulus was removed and iNOS began to degrade (Figure 2A).

To determine the physiological relevance of the SPSB1-iNOS interaction, viral-mediated short hairpin (sh) RNA technology was employed to reduce Spsb1 expression in BMDM. Spsb2−/− BMDM transduced with nonsense shRNA virus showed an induction of iNOS at 6 h post-treatment, which continued throughout the time course for both LPS and PolyI:C. Spsb1 shRNA virus, however, displayed a change in the kinetics, with expression of iNOS observed earlier, at 5 h treatment. In general, more iNOS was present in Spsb2−/− BMDM transduced with Spsb1 shRNA virus, presumably due to the earlier induction of iNOS and subsequent accumulation of protein (Figure 2B) [20].

In summary, we identified two members of the SPSB family as novel negative regulators that recruit an E3 ubiquitin ligase complex and act in concert to polyubiquitinate iNOS, resulting in its proteosomal degradation. SPSB2-deficient macrophages showed prolonged iNOS expression, which resulted in enhanced nitric oxide levels following challenge with endotoxin (LPS), gram-positive Listeria and Mycobacteria, and Leishmania parasites, all of which trigger host responses via different Toll-like receptors. The observed increase in nitric oxide production further resulted in enhanced killing of Leishmania major parasites [17]. Knockdown of SPSB1 in SPSB2-deficient macrophages resulted in greater expression of iNOS than observed with the loss of SPSB1 or SPSB2 alone (Figure 2B). Our results indicate that SPSB1 and SPSB2 co-ordinate to regulate iNOS expression. Given that SPSB1 and SPSB2 bind to the same sequence in iNOS, inhibitors that mimic the DINNN peptide should disrupt regulation by multiple SPSB proteins. It should be noted that in these experiments the level of only one SPSB protein was modified; the effects on iNOS levels of down-regulating all three relevant SPSB proteins (1, 2 and 4) in the presence of DINNN-based peptides would be expected to be even more dramatic.

It should be noted that nitric oxide is a potent chemical, which has an important role in vascular biology as well as other key physiological functions [21]. Indeed, this molecule has the distinction of having an entire journal devoted to it (www.journals.elsevier.com/nitric-oxide). The NO produced by infected macrophages to enhance pathogen killing and clearance should be confined to the site of infection. Moreover, its production will be self-limiting as it should decline once the infection is cleared and the stimulus for its production has disappeared. Thus, the higher levels of NO produced in the presence of inhibitors of the iNOS-SPSB interaction are expected to be short-lived and localised to the site of infection.

3. PEPTIDE INHIBITORS

Based on these results, we set out to design and synthesise inhibitors of the SPSB-iNOS interaction, which would be valuable tools to probe the physiological role of this interaction, as well as having therapeutic potential as anti-infective agents with a novel mechanism of action. A fragment screen was conducted [22], and the hits from this study are currently being elaborated. An in silico screen was also carried out, but the hits obtained proved to be poor inhibitors and were not pursued. Peptides, on the other hand, proved to be highly promising leads, and are the focus of this article.

As described above, peptides with the DINNN motif of iNOS represent the key interacting portion of this protein. A 13-residue peptide from the N-terminus of iNOS that encompasses this sequence had a K_{D} of 13 nM [17], while the DINNN pentapeptide had a much weaker K_{D} of ca 350 nM [23]. As the bound conformation of the peptide has a reverse turn across the DINNN sequence [18], we designed small peptides in which the native conformation was stabilized by...
bridging tethers [24, 25]. Computer modelling confirmed the potential of both the disulfide-containing octapeptide Ac-c[CVDINNNC]-NH₂ (1) (Figure 3) [26] and the head to tail lactam-bridged heptapeptide c[WDINNNβA] (2) [27], both of which were readily synthesized.

Titration of peptides 1 and 2 into SPSB2 revealed similar chemical shift perturbations in [¹H,¹⁵N]-HSQC and ¹⁹F NMR spectra [28] of SPSB2 to those caused by linear DINNN. SPR and ITC indicated that cyclic peptide 1 bound to SPSB2 with K_D values of 4 and 21 nM, respectively [26]. Moreover, the solution structure of cyclic peptide 1 determined by NMR closely matched (backbone RMSD 1.21 Å) the crystal structure of the linear peptide bound to SPSB2 [26]. Importantly, these peptides were resistant to proteolysis and stable in human plasma in vitro [26]. Moreover, unpublished data confirm that they can bind equally well to murine and human SPSB2 and 4, which is not surprising given the conservation of the iNOS binding site, but has the important implication that they should be effective against all three SPSB proteins responsible for iNOS degradation.

The disulfide-containing peptide 1 is susceptible to reduction [27]. While disulfide bridges can form in the cytoplasm under oxidative stress [29], for example during infection, cyclic peptide analogues that are stable to the normally reducing conditions of the cell cytoplasm would be likely to be more effective. While the backbone-cyclized peptide 2 eliminated the disulfide bridge, disulfides can also be replaced with thioethers [30-33], dicarba-bridges [34, 35] and di-selenides [36] to generate redox-stable analogues. Therefore, an analogue of 1 was designed in which the disulfide bridge was replaced with a thioether linkage. This cystathionine-stabilized peptide bound to SPSB2 with a K_D of 31 nM [27] and was resistant to reduction in vitro, as expected, but the thioether bridge was susceptible to oxidation by hydrogen peroxide. Even though the oxidized peptide was still able to bind to SPSB2 [27], the backbone cyclized peptide 2 was preferred.

The ability of these cyclic peptides to compete with full-length iNOS for binding to SPSB2 in a more physiological setting was confirmed. GST-SPSB2(SPRY domain), in the presence and absence of the cyclic peptides, was added to murine BMDM cell lysates containing endogenous iNOS and recovered using glutathione-Sepharose beads. Western blotting with anti-iNOS antibodies indicated that these peptides were able to compete with full-length iNOS for binding to SPSB2 [26, 27].

We also explored whether analogues of the cyclic peptide that incorporated non-peptide backbone elements would offer any enhancements in affinity or stability. Peptide mimetics incorporating several different organic linkers were designed, synthesized and characterized by NMR (¹H and ¹⁵N), SPR and ITC [37]. Three of the four mimetics synthesized bound to SPSB2 with K_D values of 29-99 nM, but their affinities were no better than the backbone-cyclized peptides and the latter were more readily synthesized, so they remained our preferred lead scaffolds.

4. PEPTIDE DELIVERY

Since our lead inhibitors were peptides, we then had to consider how to deliver them to the target cell cytoplasm. There is an extensive literature on the significant challenge of getting peptides into cells. Strategies such as backbone N-methylation and cyclization appear to be beneficial [38, 39], although each peptide presents its own challenges [40].

Coupling peptides to so-called Cell-Penetrating Peptides (CPPs) is a viable approach for experimental studies in vitro and in vivo. Arginine-rich cell-penetrating peptides such as HIV Tat, penetratin and designed oligoarginine peptides have been widely used [41], although their entry mechanism is still not entirely understood [42-44]. While CPPs are often effective as tools for delivering peptides (and other cargoes) to cells, to date, however, they have failed to progress to the clinic [45, 46].

Progress has been made in fusing cyclic peptides with cyclic cell-penetrating peptides to produce bicyclic peptides that are cell-permeable and retain the ability to recognize specific intracellular targets [47, 48]. Methods for monitoring endosomal escape of peptides coupled to CPPs are important in verifying that cargoes have been delivered to the cytoplasm rather than being trapped in endosomes. Recently, a split-complementation endosomal escape assay was described that visualizes cytosolic internalization of CPPs by fluorescence [49]. This has led to the development of new Functional Penetrating Peptides (FPPs) that offer the prospect of effective delivery to the cytoplasm.

One advantage of utilizing a CPP is that it enables the efficacy of the cargo peptide to be evaluated, at least in vitro [50]. By this means, any untoward toxicity can be identified at an early stage in the project and addressed if necessary with further modification of the cargo peptide. In the case of our peptide inhibitors of the SPSB-iNOS interaction, we instead used microinjection of the peptides [51] as a way of confirming the absence of toxicity to target cells (unpublished results).

The lack of toxicity of our cyclic peptides in several different cell lines bodes well for their therapeutic application and suggests that targeted delivery to macrophages would not be essential. Nonetheless, we are exploring ways to target these peptides to macrophages in order to ensure their
selectivity. Tissue macrophages, including those in mouse and human lung, present a number of endocytic carbohydrate-binding receptors, including macrophage galactose lectin-1 (CD301) and mannose receptor (CD206) [52, 53], which are potential targets for polymeric constructs carrying appropriate sugars such as N-acetylgalactosamine or mannose, respectively. These receptors are not expressed on surrounding cells and tissues such as epithelia, fibroblasts and blood vessels, making them attractive for specific targeting of macrophages. Synthetic procedures developed by Perrier et al., [54] will be employed to design a variety of glyco-polymer architectures for conjugation to the peptides. Living radical polymerization techniques will be employed, which enable the design of well-controlled polymeric architectures, and have shown great potential for medical applications [55]. Initial conjugation techniques will focus on active ester/amine reactions using polymers presenting a N-hydroxysuccinimide group at their α chain end, which will react with the N-termini of the peptides [56]. We will also explore other conjugation techniques, for instance reacting the thiol of an N-terminal cysteine with a maleimide chain end functional polyglycol. The architecture of the polymer can also be varied from linear to branched, as can the sequence of sugar moieties along the polymer backbone.

Macrophages undergo specific differentiation depending on the local tissue environment to produce M1 and M2 phe-nootypes [57, 58]. M1, the pro-inflammatory phenotype formed under the influence of LPS and/or IFN-γ, is involved in production of reactive oxygen species and reactive nitrogen species, promotion of the Type 1 T helper (Th1) response, and strong microbiocidal and tumoricidal activity. M2, the anti-inflammatory phenotype, is formed under the influence of interleukin-4 (IL-4), IL-13, and IL-10, and is involved in wound healing, tissue repair and remoulding and other immunosuppressive functions [57]. One challenge of targeting via glycopolymer conjugation, therefore, will be to direct the peptide conjugates to M1 macrophages without compromising their viability [59].

Alternative delivery systems exist. For example, M1 macrophages could be selectively targeted by model hybrid lipid-latex nanoparticles bearing phagocytic signals [60]. Liposomes incorporating arginine derivatives were able to encapsulate doxorubicin and deliver it to both M1 and M2 macrophages [61].

5. PEPTIDE DISPOSITION

Whichever construct proves to be effective in being taken up by macrophages, and then delivering the peptide cargo to the cytoplasm of those cells, it will be important to monitor the pharmacokinetics, pharmacodynamics and distribution in vivo of that peptide. Peptides have traditionally been regarded as poor therapeutic candidates because of their expected short half-lives in vivo and their susceptibility to proteolysis [62, 63]. However, we note that the current cyclic peptide leads are resistant to proteolysis, have excellent redox stability, and are not degraded in plasma [27].

Moreover, because they are small and readily synthesized, cost-of-goods is unlikely to be an issue should they progress to clinical development.

They are not expected to be orally bioavailable, but there are several viable options for delivery, including buccal [64, 65] and pulmonary [66] administration, the latter being particularly relevant to the treatment of respiratory infections such as tuberculosis. Slow-release biodegradable polymer matrices prepared from poly-lactic-co-glycolic acid, which degrade slowly and release peptide continuously for up to six months [66, 67] could also be employed.

Peptide (or peptide conjugate) distribution in vivo should also be monitored. In the case of the potassium channel blocking peptide ShK-186 (dalazatide), for example, SPECT/CT data on an 111In-DOTA-conjugate of the peptide showed that the peptide persisted at the site of sub-cutaneous injection for up to a week, accounting for the fact that a single dose of ShK-186 every 2-5 days was as effective as daily administration in delayed-type hypersensitivity, chronic relapsing-remitting experimental autoimmune encephalomyelitis and pristane-induced arthritis rat models [68]. Another venom-derived peptide, HsTX1[R14A] showed similar behavior [69, 70]. In both of these cases there was a significant disconnect between the pharmacokinetics, which reflected rapid renal clearance, and pharmacodynamics, which showed a sustained in vivo efficacy. Monitoring the in vivo distribution and lifetime will therefore be important in evaluating where and when to deploy the lead peptide inhibitor of the SPSB-iNOS interaction.

CONCLUSION

This article has outlined the rationale for exploring inhibitors of the SPSB-iNOS interaction as new antibiotics that act by enhancing the host immune response rather than directly by attacking the infectious organism. Small cyclic peptides are currently the best leads, and efforts are focused on delivering them to the cytoplasm of macrophages. Many of the challenges often faced by peptides as potential therapeutics do not apply to these peptides because of their small size, high stability and ease of synthesis. Our focus is now on efficient delivery to the site of action in the cytoplasm of M1 macrophages.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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