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A Peptidyl Inhibitor that Blocks Calcineurin-NFAT Interaction and Prevents Acute Lung Injury

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Abstract

Acute respiratory distress syndrome (ARDS) is an inflammatory lung disease with a high morbidity and mortality rate, for which no pharmacologic treatment is currently available. Our previous studies discovered that a pivotal step in the disease process is the activation of the nuclear factor of activated T cells (NFAT) c3 in lung macrophages, suggesting that inhibitors against the upstream protein phosphatase calcineurin should be effective for prevention/treatment of ARDS. Herein we report the development of a highly potent, cell-permeable, and metabolically stable peptidyl inhibitor, CNI103, which selectively blocks the interaction between calcineurin and NFATc3, through computational and medicinal chemistry. CNI103 specifically inhibited

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[†]PD and MK contributed equally to this work. DP, JWC, MK, and PD conceived the project. DP and PD designed the computational, combinatorial library, and biochemical experiments. PD and AK carried out the compound synthesis and in vitro characterization. MK and JWC designed the mouse ALI and toxicity studies and MK, TSN, LR, and SC performed ALI model experiments. ABH and JKL designed or performed mass spectrometry imaging experiments. HWL and MK performed the biodistribution and immune cell entry studies. ZQ and PD designed and scientists at BioDuro Inc. performed the in vitro metabolism and in vivo pharmacokinetics of CNI103. DP, MK, PD, and JWC analyzed the data and wrote the manuscript, which was approved by all authors.

Supporting Information. Peptide structures, their molecular formula strings, quality control data, and additional experimental data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare the following competing financial interests: A patent application has been filed on CNI103 and related compounds; DP is a cofounder and shareholder of Entrada Therapeutics.

calcineurin signaling *in vitro* and *in vivo* and exhibited a favorable pharmacokinetic profile, broad tissue distribution following different routes of administration, and minimal toxicity. Our data indicate that CNI103 is a promising novel treatment for ARDS and other inflammatory diseases.

Graphical Abstract

Keywords

Acute respiratory distress syndrome; Calcineurin inhibitor; Cell-penetrating peptide; peptide therapeutics; Protein-protein interaction

INTRODUCTION

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS) are complications of diverse conditions including systemic inflammatory disorders, direct injuries, and infections in the lungs including those caused by SARS-CoV-2, the coronavirus responsible for the current global pandemic. ALI/ARDS is quite common (~200,000 ARDS cases/year in the US before the current pandemic) and associated with high mortality (~40%).¹ Currently, conservative supportive care is the only treatment option and standard treatments for ARDS include non-injurious mechanical ventilation strategies, prone positioning, conservative fluid management, and antibiotics for infections. Although these measures have improved the survival, there is no specific pharmacologic treatment based on the molecular pathophysiology of ARDS. However, since the first report of ARDS almost 50 years ago, many pharmacologic agents have been evaluated for treating ARDS.¹⁻³ To date, a few have shown promise in early stages of investigations, but none have been effective in randomized multicenter phase II/III clinical trials to treat or prevent ARDS, including the most recently evaluated β_2 agonist therapy,^{4,5} statins,⁶ early neuromuscular blockade,⁷ vitamin D3,⁸ and mesenchymal stem cell therapy.⁹ Failure of these drug approaches is probably due to the heterogeneity and redundancy of the mechanisms underlying the pathogenesis of ALI/ARDS.

ALI/ARDS pathophysiology is characterized by a disrupted pulmonary microvascular endothelium and alveolar epithelium, coupled with impaired fluid removal from the alveolar space, and hypoxemic respiratory failure.¹⁰ Increased neutrophilic infiltration and the release of pro-inflammatory cytokines such as TNF α and IL-6 result in diffuse alveolar damage and severe hypoxemia.¹¹ Extensive free radical production by recruited leukocytes in ARDS is thought to overwhelm endogenous anti-oxidants and causes oxidative cell damage.¹² We previously showed that activation of nuclear factor of activated T cells cytoplasmic member

3 (NFATc3) regulates the inflammatory phenotype of lung macrophages that contributes to the development of sepsis-induced ALI in mouse models.¹³ Genetic ablation of NFATc3 significantly attenuates the development of ALI in mice and is associated with improved pulmonary barrier function, oxygenation, and better survival.¹⁴ These data suggest that pharmacological inhibition of the NFATc3 function should provide an effective therapy for ALI and ARDS.

Because transcription factors such as the NFATs are challenging targets for current drug modalities, we turned our attention to the proteins that regulate the activity of NFATs. In T cells, macrophages, and other cell types, the primary mechanism of NFAT activation is mediated by the mobilization of calcium and activation of calmodulin-dependent serine/threonine protein phosphatase, calcineurin (CN), which dephosphorylates cytosolic proteins including NFATs.¹⁵ The dephosphorylated NFATs translocate into the nucleus and up-regulate the expression of proinflammatory genes. However, existing calcineurin inhibitors such as cyclosporine A (CsA) and FK506, while highly effective for preventing allograft rejection in organ transplantation patients, have not been used for prevention or treatment of ARDS, likely because of their severe side effects, particularly nephrotoxicity.¹⁶ CsA and FK506 function by first engaging endogenous proteins, cyclophilin and FKBP, respectively.¹⁷ The resulting CsA/cyclophilin and FK506/FKBP complexes subsequently bind to CN and physically block the access of *all* protein substrates to CN. Binding and inhibition of the cyclophilin/FKBP function per se likely also result in significant toxicities. Additionally, CsA and FK506 enter mammalian cells by passive diffusion, making them active against most cell types including T cells. These properties render CsA and FK506 less desirable options for prevention or treatment of ARDS caused by pathogenic infections (e.g., SARS-CoV-2) due to their serious side effects and potential to reduce adaptive host immunity.¹⁸ Consequently, there has been significant interest in developing direct CN inhibitors that do not engage cyclophilin, FKBP, or other endogenous proteins. Several such small-molecule CN inhibitors were recently reported, including thiol-reactive covalent inhibitors INCA-6^{19,20} and MCV1/2²¹, as well as competitive, active site-directed inhibitors 2j,²² NCA-01,²³ and CN585.²⁴

Cell-permeable peptides represent an emerging class of therapeutic modalities, which are ideally suited for targeting challenging proteins, such as those involved in intracellular protein-protein interactions (PPIs).^{25–27} In the case of CN, previous studies identified a conserved sequence motif among NFATs and a small number of other CN substrates, PxIxIT (where x is any amino acid), which specifically interacts with a substrate-docking site on CN.²⁸ Since the CN-NFAT interaction is essential for efficient dephosphorylation and activation of NFATs,¹⁵ an inhibitor against this PPI would serve as an effective immunosuppressant, without many of the off-target side effects of CsA and FK506. Through screening of a peptide library, Aramburu et al. discovered a tetradecapeptide, GPHPVIVITGPHEE (VIVIT), which binds to the NFAT-docking site of CN with a 25-fold higher affinity than the naturally occurring PxIxIT motif.²⁸ Conjugates of the VIVIT peptide with cationic cell-penetrating peptides (e.g., R₁₁) have demonstrated *in vivo* efficacy in several disease models, including cardiovascular diseases,^{29–31} transplantation,³² colitis,³³ and others.^{34–38} While demonstrating the myriad potential benefits of a CN inhibitor that does not affect its phosphatase activity,³⁹ linear peptides such as VIVIT are unlikely to be

clinically relevant due to extensive metabolic liabilities. We previously modified the VIVIT sequence ($K_D \sim 500$ nM for CN) by replacing the two valine residues with *tert*-leucine (Z) and obtained an improved peptidyl inhibitor ZIZIT ($K_D = 50$ nM).⁴⁰ Further replacement of the C-terminal proline residue with a cis-proline analog, 2,2-dimethyl-1,3-thiazolidine-4-carboxylic acid (cisPro), resulted in ZIZIT-cisPro as a highly potent CN inhibitor ($K_D = 2.6$ nM). Conjugation of ZIZIT to a highly active cyclic cell-penetrating peptide (CPP9)⁴¹ resulted in a biologically active CN-NFAT inhibitor (CPP9-ZIZIT) which inhibited NFATc3-dependent inflammatory gene expression in macrophages and prevented lipopolysaccharide (LPS)-induced pulmonary inflammation and edema in mouse models.¹⁴ However, CPP9-ZIZIT is not an ideal candidate for clinical development due to its proteolytic instability (serum $t_{1/2} \sim 6$ h) and suboptimal potency. Meanwhile, small-molecule inhibitors have been reported against the CN-NFAT interaction, but lack the requisite specificity and affinity.⁴² In this work, we optimized the structure of CPP9-ZIZIT by employing *in silico* modeling and combinatorial library approaches. These efforts led to a cell-permeable, highly potent ($K_D = 16$ nM), and metabolically stable CN inhibitor (serum $t_{1/2} > 24$ h), CN1103, with favorable pharmacokinetics and *in vivo* efficacy for preventing LPS-induced ALI in mouse models.

RESULTS AND DISCUSSION

Overall Strategy for CN Inhibitor Optimization.

The discovery of ZIZIT-cisPro (Figure 1) as a potent CN inhibitor ($K_D = 2.6$ nM) demonstrated the feasibility of designing highly potent and selective inhibitors against the CN-NFAT interaction. The availability of highly active and metabolically stable cyclic CPPs enabled the effective delivery of peptidyl drugs into the cytosol of mammalian cells.^{41,43,44} However, ZIZIT-cisPro has limited metabolic stability and is a synthetic challenge because the cisPro residue is incompatible with the standard solid-phase peptide synthesis chemistry. Once incorporated into a peptide, cisPro sterically blocks further elongation of the peptide chain. Moreover, cisPro is labile under the acidic condition required for peptide deprotection. We therefore focused our optimization efforts on improving the metabolic stability and synthetic accessibility of ZIZIT-cisPro. Inspection of the structure of ZIZIT-cisPro (Figure 1) suggests that its N- (Gly-Pro-His-Pro) and C-terminal sequences (Gly-cisPro-His-Glu-Glu) are likely responsible for the observed proteolytic liability, whereas the core CN-binding motif, Tle-Ile-Tle-Ile-Thr (ZIZIT), should be more protease resistant, thanks to the presence of multiple β -branched amino acids (e.g., Tle). We envisioned that replacement of the N-terminal tetrapeptide (GPHP) with a non-peptidyl moiety (e.g., an acyl group) would eliminate any proteolytic liability associated with this segment. With a wide variety of available acyl groups to choose from, one might identify a “small molecule” that fits into the hydrophobic pocket occupied by Pro⁴ of ZIZIT-cisPro (Figure 1A). Replacement of the C-terminal pentapeptide (GPHEE) with a “small molecule” is more challenging, as the pentapeptide-binding site lacks any major pocket and the C-terminal region forms an intricate intermolecular hydrogen bond network with CN. Therefore, we opted for a combinatorial library approach to optimize the C-terminal sequence. In brief, the pentapeptide was replaced with a library of short peptides enriched in D-amino acids and the resulting peptide library was screened for binding to CN (Figure 1B). We anticipated that some of the library hits might contain D-amino acids in the C-terminal region and have

improved proteolytic stability over ZIZIT-cisPro, while maintaining the CN-binding affinity of the latter. Any optimized CN-binding peptide would be conjugated to a cyclic CPP for intracellular delivery.

Replacement of the N-Terminal Sequence of ZIZIT with an Acyl Group.

To facilitate concentration determination and binding analysis by fluorescence polarization (FP), a dipeptide Tyr-Lys was added to the C-terminus of ZIZIT and the lysine side chain was labeled with fluorescein isothiocyanate (FITC). The resulting peptide, Ac-GPHPZIZITGPHEEYK(FITC)-NH₂ (Table 1, peptide **1**), bound to CN with a K_D value of 92 nM. Next, the N-terminal four residues (Ac-GPHP) were replaced with benzoyl, cyclopentylcarbonyl, cyclohexylcarbonyl, acetyl, or formyl group and the resulting peptides (Table 1, peptide **2–6**) were subjected to *in silico* rigid receptor/flexible ligand docking against an ensemble of molecular dynamics (MD)-generated CN conformations associated with a fixed core sequence (ZIZITGP) in Schrödinger *Glide*.^{45,46} The benzoyl, cyclopentylcarbonyl, cyclohexylcarbonyl groups were intended to mimic the prolyl residue at position 4 of ZIZIT (Figure 1), whereas the acetyl and formyl groups were included as negative controls to validate the *in silico* model. *In silico* docking suggested that the benzoyl group was most effective, with an approximated binding energy of –12.8 kcal/mol for peptide **2**, whereas the formyl group was least effective with an approximated binding energy of –9.5 kcal/mol for peptide **6** (Table 1). FP analysis confirmed the benzoyl and cyclohexylcarbonyl groups as the most effective surrogates of the tetrapeptide (K_D values of 224 and 207 nM for peptides **2** and **4**, respectively). The importance of the benzoyl group is illustrated by a >20-fold reduction in the CN-binding affinity when it was replaced by a formyl or acetyl group. Remarkably, substitution of a simple acyl group (e.g., benzoyl) for the tetrapeptide only reduced the CN-binding affinity by ~2-fold.

The benzoyl group was selected for further optimization because of the commercial availability of a larger number of benzoic acid derivatives. Additional *in silico* docking analysis was performed for a focused library of halogenated and/or alkylated benzoic acid derivatives (total 61 compounds, Table S1), leading to the identification of five benzoyl derivatives with predicted improvement in CN binding affinity over the benzoyl group (Table 1). FP analysis of the corresponding acyl-ZIZITGPHEEYK(FITC)-NH₂ peptides (Table 1, peptides **7–11**) confirmed that they all have higher CN-binding affinity than the benzoylated peptide (K_D values of 32 to 189 nM). Peptide **9**, which contains an N-terminal 2,3-dichlorobenzoyl (Dcb) group (K_D = 41 nM), was selected for C-terminal optimization. Examination of the MD-refined docking pose revealed that the benzoyl group fits snugly into the proline-4 binding pocket, with the two chlorine atoms facing the solvent (Figure 2). It appears that the presence of *ortho*-substitution twists the benzoyl carbonyl moiety out of the benzene plane, allowing the benzene ring to optimally insert into the hydrophobic pocket.

Optimization of the C-Terminal Sequence of ZIZIT by Library Screening.

The C-terminal pentapeptide (GPHEE) contributes significantly to the overall CN-binding affinity, as its removal decreased the binding affinity by 17-fold (K_D = 700 nM for peptide **12**; Table 2). To replace the pentapeptide with a more proteolytically stable and potentially

tighter binding sequence, we designed a one bead-one compound (OBOC) peptide library⁴⁷ in the form of Dcb-ZIZIT- X_n -BBRRM-resin, where X_n represents a random peptide sequence of 1 to 4 residues and B is β -alanine (Figures 1 and S1). Each random position (X^1 to X^4) was constructed with a set of 28 amino acids, including 11 proteinogenic amino acids (Ala, Arg, Asp, Gln, Gly, His, Ile, Pro, Ser, Trp, and Tyr), 6 unnatural α -L-amino acids [4-fluorophenylalanine (Fpa), 2-naphthylalanine (Nal), norleucine (Nle), ornithine (Orn), phenylglycine (Phg), and pipecolic acid (Pip)], 7 α -D-amino acids (ala, asn, glu, leu, lys, phe, thr), and 4 β -amino acids [(1*S*,2*R*)-2-aminocyclopentane carboxylic acid (Acp), β -alanine (β Ala), β -D-homoalanine (bha), and isoaspartic acid (Ias)]. The library has a theoretical diversity of 6.5×10^5 unique compounds and was synthesized on 1 g of TentaGel resin (90 μ m), as detailed in the Experimental Section. The library (200 mg or $\sim 600,000$ compounds) was screened for binding to a biotinylated glutathione-S-transferase-calcineurin (GST-CN) fusion protein and the positive beads were stained by incubation with a streptavidin-alkaline phosphatase conjugate (SA-AP) and 5-bromo-4-chloro-3-indolyl phosphate.⁴⁸ Forty-nine most intensely colored beads were isolated from the library and the peptides released from these beads were individually analyzed by MALDI-TOF mass spectrometry (Figure S2), giving 46 unambiguous, complete sequences (Table S2). Interestingly, all of the selected peptides contained four random residues (the maximum peptide length for any library sequence), indicating that all four residues are essential for optimal CN binding. Inspection of the selected sequences revealed that CN strongly prefers an L-amino acid at the X^1 position (which is immediately C-terminal to the ZIZIT motif), especially glutamine and tyrosine and, to a lesser extent, aspartic acid and histidine (Figure 3). It also has a strong selectivity for histidine, tyrosine, or D-glutamic acid at the X^2 position. At the X^3 and X^4 positions, CN has a general preference for aromatic hydrophobic residues (especially Nal), although a variety of other amino acids (including D-amino acids) are tolerated.

Five representative sequences were resynthesized, labeled at their C-termini with a β Ala-Lys(FITC) moiety, and tested for CN binding by FP (Table 2, peptides **13–17**). All five peptides bound to CN with high affinity (K_D values of 4.7 to 64 nM; Figure S3). Peptides **13** (which has a C-terminal sequence of Tyr-glu-Fpa-His and a K_D value of 12 nM) and **14** (Gln-His-leu-Ser; K_D = 9.1 nM) were selected for further studies because of their high potency, relatively hydrophilic sequences, and the presence of a D-amino acid in the C-terminal region (which should improve their proteolytic stability). Peptide **15**, despite its excellent potency (K_D = 4.7 nM), was not selected for further evaluation because it is highly similar to peptide **14** but has a more hydrophobic histidine instead of a serine at the C-terminus.

Generation of Cell-Permeable CN Inhibitors by Conjugation to Cyclic CPP.

To render peptides **13** and **14** cell-permeable, a cyclic CPP (CPP9) was covalently attached to their C-termini through an amide bond and a β Ala-Lys linker, resulting in peptides **18** (CNI88) and **19** (CNI93), respectively (Figure 4A). CPP9 is a highly active and metabolically stable CPP, having a cytosolic delivery efficiency of 62% (100% efficiency is defined as resulting in equal cargo concentration in the cytosol and the extracellular medium).⁴¹ It enters the cell by endocytosis and subsequently exits the early endosome into

the cytosol by inducing budding and collapse of small vesicles from the endosomal membrane.^{41,49,50} To ascertain that CNI88 and CNI93 retain the ability of binding to the NFAT-docking site on CN, they were tested for competition with FITC-labeled peptide **14** for binding to CN in an FP-based competition assay. CNI93 dose-dependently inhibited the binding of peptide **14** to CN, with an IC₅₀ value of 630 nM (Figure 4B). Under the same conditions, CNI88 had an IC₅₀ value of ~2.1 μ M. CNI93 was selected as the lead compound for further characterization because of its higher potency relative to CNI88. In a direct binding assay by FP, CNI93 showed a K_D value of 75 nM for CN (Figure S3), indicating that conjugation with CPP9 reduced the CN-binding affinity of peptide **14** by ~8-fold.

CNI93 has excellent serum stability; incubation of CNI93 in human serum at 37 °C for 24 h resulted in no significant degradation (<10%; Figure 4C). Under the same condition, ZIZIT-cisPro was degraded with a half-life of ~6 h, while the VIVIT peptide was completely degraded within 6 h ($t_{1/2}$ ~1 h). The aqueous solubility of CNI93 was assessed by measuring turbidity in solution. The peptide was serially diluted from a DMSO stock into PBS (pH 7.4) containing 5% DMSO and the optical density of the resulting solutions were measured at 780 nm. In the presence of 5% DMSO, CNI93 did not significantly increase the turbidity up to 100 μ M (the highest concentration tested), suggesting that CNI93 has an aqueous solubility of at least 100 μ M (Figure S4).

The cell permeability of CNI93 was estimated by labeling it with naphthofluorescein (NF) and quantitating the mean fluorescence intensity of HeLa cells treated with the peptide by flow cytometry.⁵¹ NF is a pH-sensitive dye (pK_a = 7.8) and is non-fluorescent in the acidic environments of endosomes/lysosomes. The NF fluorescence intensity of the treated cells therefore reflects the amount of the peptide that has reached the cytosol and nucleus of the cells. Flow cytometry analysis revealed that CNI93 entered HeLa cells with a relative cytosolic delivery efficiency of 109% (relative to that of CPP9, which is defined as 100%; Figure 4D). Live-cell confocal microscopic analysis confirmed that CNI93 reached the interior of HeLa cells (data not shown). Finally, CNI93 was assessed for general cytotoxicity against HeLa (cervical cancer) and H441 (lung cancer) cells by using the viability assay. Treatment of the cells with up to 25 μ M CNI93 for 72 h resulted in no significant loss of cell viability (Figure S5).

CNI93 Inhibits NFATc3 Nuclear Translocation and Target Gene Expression.

We have previously demonstrated that treatment with LPS causes NFATc3 translocation from cytoplasm to nucleus and regulation of NFATc3 target genes in macrophages.¹⁴ Since nuclear translocation of NFATs requires prior dephosphorylation by CN, we tested whether CNI93 can block the activation and nuclear translocation of NFATc3 in macrophages. Lung macrophages derived from collagenase treated lung tissue from healthy mice were pretreated with biotinylated CNI93 or a non-binding control peptide, CPP9-VAVAA (peptide **20**), and then stimulated with LPS (or PBS). The intracellular localization of NFATc3 and biotinylated CNI93 (or CPP9-VAVAA) was visualized by immunofluorescence staining with Alexa Fluor® 488 conjugated anti-NFATc3 antibody and Alexa Fluor® 594-conjugated streptavidin, respectively. In cells treated with CPP9-VAVAA and PBS, NFATc3 was predominantly localized in the cytoplasm, whereas LPS stimulation resulted in almost

complete nuclear localization of NFATc3 (Figure 5A). In contrast, pretreatment of the cells with 1 μ M CNI93 completely blocked LPS-induced nuclear translocation of NFATc3 (Figure 5B).

We next examined the effect of CNI93 on NFATc3 target gene expression in macrophages. Activated NFATc3 upregulates the expression levels of tumor necrosis factor- α (TNF α), iNOS, IL-6, and CCR2 in macrophages.^{13,14} Lung macrophages from healthy mice were untreated or treated with different concentrations of CPP9-VAVAA, CsA or CNI93 and then stimulated with LPS, and the amount of TNF α and IL-6 secreted was quantified by ELISA. Stimulation of untreated cells with 100 ng/mL LPS for 8 h resulted in 10-fold increases in both TNF α and IL-6 production (Figure 5C and D). Prior treatment of macrophages with CNI93 dose-dependently decreased LPS-induced TNF α secretion by 17–39% and IL-6 production by 22–39%. As a positive control, treatment with 10 μ M CsA reduced the TNF α production by 24% and IL-6 by 37%. These results indicate that CNI93 is comparable to and perhaps slightly more potent than CsA as an anti-inflammatory agent. Note that inhibition of TNF α and IL-6 production was not complete even at high concentrations of CNI93 or CsA (10 μ M). This is likely because LPS also activates other inflammatory signaling pathways (e.g., NF κ B pathway), which are not directly inhibited by CN inhibitors.⁵² CPP9-VAVAA (10 μ M) also caused a small reduction in cytokine secretion, probably because of its general cytotoxicity and reduced cell viability.

***In vivo* Uptake of CNI93 by Lung Cells.**

Tetramethyl rhodamine (TMR)-labelled CNI93 (CNI93^{TMR}) was instilled into both nostrils of anesthetized C57BL/6 mice (5 mg/kg in 25 μ L of vehicle). After 1, 2, 4, 16 and 24 h, the mice were euthanized and lungs were digested in collagenase and DNase to obtain single cell suspension and analyzed by flow cytometry. The CNI93^{TMR} concentration in the bronchoalveolar lavage fluid (BALF) peaked at ~2 h, followed by slow decline and eventual clearance at between 16 and 24 h (Figure 6A). TMR-positive lung leukocytes (CD45⁺ CD31⁻ CD326⁻), epithelial (CD45⁻ CD31⁻ CD326⁺) and endothelial cells (CD45⁻ CD31⁺ CD326⁻) were compared at different time points. Fifty to 80% of total leukocytes were TMR positive at 4 h and the level declined gradually by 24 h (Figure 6B). Interestingly, lung leukocytes showed much greater uptake of CNI93 (by 3- to 5-fold) compared to epithelial cells, probably due to the higher endocytosis rates of leukocytes (e.g., macrophages) compared to structural lung cells. It is worth noting that the blood plasma levels of CNI93^{TMR} were negligible throughout the 24-h time course (data not shown). Total lung cells from untreated and CNI93 treated mice showed similar lactate dehydrogenase levels as assayed by CytoTox 96 indicating no cytotoxic effects of CNI93 (data not shown). In a parallel experiment, cytospin slides of total bronchoalveolar lavage cells which were stained by Hema 3.0 showed no neutrophil infiltration upon peptide treatment, indicating that the peptide itself does not cause neutrophilic inflammation (Figure S6).

CNI93 Prevents LPS-induced ALI.

Next, we examined the protective effect of CNI93 in LPS-induced ALI mouse models. Mice were pretreated with CPP9-VAVAA or CNI93 (5 or 10 mg/kg) given by intranasal insufflation (i.n.) and after 2 h, administered with saline or LPS [10 mg/kg, intraperitoneal

(i.p.)] and euthanized at 16 h for analysis. As expected, LPS challenge of CPP9-VAVAA treated mice increased the total protein in BALF by ~2-fold and TNF α and IL-6 levels in BALF by 3- to 5-fold (Figure 7A, B, and C). In contrast, pretreatment with CNI93 (10 mg/kg) largely prevented the LPS-induced TNF α and IL-6 secretion in BALF (Figure 7A, B) and extravasated BALF protein levels (Figure 7C). Plasma levels of TNF α and IL-6 were also significantly lower in CNI93-treated mice than mice pretreated with CPP9-VAVAA (Figure 7D, E). In addition, CNI93 significantly reduced the lung wet-to-dry ratios (Figure 7F) and the number of neutrophils in the BALF (Figure 7G) compared to the control peptide. These observations clearly demonstrate the protective effect of CNI93 against systemic LPS-induced ALI in the mouse model. However, *in vivo* efficacy required a relatively high dose for CNI93 (10 mg/kg), as mice treated with a lower dose (5 mg/kg i.n.) did not show significant reduction in LPS-induced BALF cytokines and other ALI parameters (data not shown).

Evaluation of Acute Toxicity of CNI93.

Acute toxicity was determined after single intraperitoneal or intranasal injection of CNI93 in C57BL/6 mice. Mice of either sex, administered with CNI93 at 50 mg/kg (i.p.) showed no change in physical appearance, body temperature, self-grooming and respiratory function measured in terms of pulmonary arterial oxygen saturation compared to untreated controls (Figure S7). In addition, mice treated with CNI93 (50 mg/kg i.p. or 10 mg/kg i.n.) showed no significant loss of body weight or changes in growth rates compared to untreated mice or mice treated with the control peptide through one week of observation (Figure S8).

Further Optimization of CN Inhibitor.

The 8-fold lower CN-binding affinity of CNI93 than peptide **14** indicates that the CPP moiety interferes with CN binding. To minimize the interference, we replaced the β -Ala-Lys linker in CNI93 with a longer and more flexible linker, miniPEG-Lys, to produce peptide **21** (CNI95, Table 3). CNI95 showed ~4-fold higher potency than CNI93 in the FP-based competition assay, for inhibition of the binding of peptide **14** to CN (IC_{50} = 150 nM). When labeled at the C-terminus with FITC and analyzed by FP, CNI95 bound to CN with a K_D value of 17 nM. Like CNI93, CNI95 has excellent cell-permeability and proteolytic stability, with a $t_{1/2}$ >24 h in human serum at 37 °C. Surprisingly, CNI95 showed no significant biological activity in either the *ex vivo* assay (LPS-induced production of TNF α or IL-6 by primary mouse lung macrophages) or the mouse ALI model (Figure S9). It was later discovered that CNI95 forms microscopic aggregates when added into growth medium, which were visible during fluorescence microscopy (data not shown). We hypothesized that the CPP9 sequence likely improved the solubility of CNI93; in the presence of a longer linker (as in CNI95), however, the exposed hydrophobic CN ligand (peptide **14**) may interact with each other and/or serum components to form aggregates.

To improve the aqueous solubility of CNI95, we explored the possibility of replacing some of the CN-binding residues with more hydrophilic ones, without compromising the overall binding affinity for CN. *In silico* docking suggested that substitution of a 2,3-dichloroisonicotinyl (Dcn) group for the N-terminal Dcb group should retain most of the binding free energy (Docking score = -13.8 kcal/mol). The docked pose for Dcn

recapitulated the same interactions as observed for Dcb, but the ring nitrogen was expected to improve aqueous solubility by hydrogen bonding with solvating water molecules, as observed for other pyridinyl-substituted peptides.⁵³ This prediction was confirmed experimentally, as the Dcn-containing peptide (**22**) exhibited only a slightly increased IC₅₀ value in the FP-based competition assay (180 nM vs 150 nM for CNI95; Table 3). We next explored replacing the two Tle residues with β -hydroxyvaline (Hva). We previously showed that substitution of Tle for Val at these two positions increased the CN-binding affinity by an order of magnitude,⁴⁰ likely because the *tert*-butyl group of Tle ensures that one or two methyl groups always face the CN surface and make hydrophobic interactions with the latter (e.g., Val-328), regardless of the side chain conformation. We felt that Hva might maintain the hydrophobic interaction through its two remaining methyl groups, while pointing its hydroxyl group into the solvent to enhance solubility. *In silico* modeling suggested that substitution of Hva for the N-terminal Tle is detrimental (Docking score = -12.0 kcal/mol for peptide **23**) but well tolerated for the second Tle (Docking score = -13.9 kcal/mol for peptide **24**). Based on these predictions, we synthesized peptide **25** by combining a Dcn at the N-terminus and a Hva in place of the second Tle and named it “CNI103” (Table 3). CNI103 showed improved aqueous solubility while largely maintaining the potency of CNI95, with an IC₅₀ value of 220 nM in the FP-based competition assay and a K_D value of 16 ± 2 nM for CN in the direct FP assay (Figure 8A). CNI103 was readily soluble in PBS (pH 7.4) at 50 μ M and caused slight increase in turbidity at 100 and 200 μ M concentrations (Figure S4). Notably, CNI103 did not inhibit the catalytic activity of CN toward a low-molecular-weight substrate, *p*-nitrophenyl phosphate; rather, it stimulated the CN activity by ~2-fold (Figure S10). This phenomenon has also been observed for CsA/cyclophilin and FK506/FKBP complexes¹⁷ and indicates that CNI103 binds to a site other than the active site.

Attempts to further optimize CNI103 were unsuccessful. Replacement of the first isoleucine residue of the ZIZIT motif with L-cyclohexylglycine (Chg) substantially increased the CN-binding affinity (by ~5-fold), but the resulting peptide (**26**) was markedly less soluble (Table 3). We also prepared 4 peptides containing alternative residues within the C-terminal region of CNI95 (Table 3). Of the peptides, only substitution of Arg for Ser was neutral with respect to potency (peptide **27**, IC₅₀ = 190 nM). Replacement of D-Leu with D-Val or Gln with Asn or Asp all decreased the potency (IC₅₀ 250 nM for peptides **28–30**). In a cell-based assay for NFAT activity in Jurkat cells, none of these compounds performed better than CNI103 (data not shown). We therefore chose CNI103 for further evaluation.

Anti-Inflammatory Activity of CNI103 Ex Vivo.

CNI103 was evaluated for its efficacy in reducing the secretion of inflammatory cytokines by LPS-stimulated macrophages. Primary mouse lung macrophages were pretreated with for 4 h with increasing concentrations (0–10 μ M) of CNI103, CsA (positive control), or CPP9-VAVAA (negative control) and then stimulated with LPS (100 ng/mL) for 8 h. CNI103 resulted in significant, dose-dependent reduction in the secreted TNF α and IL-6 levels (Figure 8C and 8D). Comparison of the dose response curves of CNI103 and CNI93 (Figure 5C and 5D) suggests that CNI103 is ~10-fold more potent than CNI93, consistent with the 5-fold higher CN-binding affinity of the former. Under the conditions of this assay, CNI103

is also substantially more effective than CsA in preventing inflammatory cytokine production (by 10- to 100-fold). For example, 1 μ M CNI103 resulted in greater reduction in TNF α and IL-6 levels than 10 μ M CsA (Figure 8C and 8D). To ensure that the observed biological activity was not a result of off-target cytotoxicity, we evaluated the general cytotoxicity of CNI103 with HeLa and mouse primary lung macrophage cells. Similar to CNI93 and CNI95, CNI103 did not cause significant loss of cell viability until 25 μ M (Figure S5).

In Vitro Metabolism of CNI103.

Since metabolic instability is frequently a key limitation of peptidyl therapeutics (especially for linear peptides), we performed extensive analyses to assess the stability of CNI103 in different biological environments. CNI103 was found to be remarkably stable in all of the tests we have conducted. Incubation of CNI103 in human serum for 24 h at 37 °C resulted in no significant degradation, while Tat, a first-generation linear CPP, was completely degraded within 30 min under the same condition (Figure 8B). Similarly, CNI103 was completely stable during a 2-h incubation with mouse hepatocytes (Table S3), a simulated gastric fluid (Table S4), or a simulated intestinal fluid (Table S5). We also incubated HCT116 cell spheroids (derived from human colon cancer) with 5 μ M CNI103 for 4 or 24 h and imaged the organoids (after exhaustive washing) by MALDI-TOF-TOF mass spectrometry.⁵⁴ The mass spectra were analyzed for intact CNI103 as well as potential proteolytic degradation fragments. The results indicated that CNI103 entered the spheroids in a time-dependent manner and accumulated in the cells, but no significant degradation product was observed after 24 h of incubation, suggesting that CNI103 is also stable in the intracellular environment (Figure S11). Finally, the liver microsome stability of CNI103 was assessed with mouse liver microsomes in the absence and presence of NADPH. Time-dependent degradation of CNI103 was observed in the presence of NADPH (up to 25% after 1 h), suggesting that CNI103 is subjected to cytochrome P450-mediated metabolism (Table S6).

In Vivo Pharmacokinetics (PK) and Biodistribution of CNI103.

BALB/c mice (n = 4) were administered intravenously (i.v.) with CNI103 dissolved in sterile PBS (5 mg/kg). At 5 min, 15 min, 30 min, 2 h, 4 h, and 8 h post dosing, blood samples (30 μ L) were collected and the plasma CNI103 concentration was determined by LC/MS/MS analysis. Interestingly, CNI103 displayed an unusual plasma PK profile, which prevented the determination of conventional PK parameters (Figure 9A and Table S7). Immediately after injection, the plasma concentration rapidly declined with an initial clearance $t_{1/2}$ 15 min. At 2 and 4 h, the plasma levels of CNI103 were undetectable by the LC-MS/MS method, which has a lower limit of quantification of 5 nM. After 8 h, however, the plasma concentrations of CNI103 returned to detectable levels, with an average of 13.5 ± 2.5 nM. This suggests that CNI103 was rapidly adsorbed by mouse tissues and/or internalized by mouse cells and an equilibrium was likely reached between the plasma and body tissues during the elimination phase. To test this notion, we extracted the CNI103 from major organs (after 8 h) and quantitated the tissue concentrations of CNI103 by LC/MS. Significant concentrations of CNI103 (15–40 nM) were found in the liver, kidney, and spleen but minimal in muscle and brain (Figure 9B). It should be noted that these concentrations are likely underestimated, as the peptide extraction yields have not been

established and are <100%. CNI103 metabolites were also detected in plasma, liver, kidney, and spleen but have not been structurally elucidated. Additionally, we labeled CNI103 with Cyanine-5 (CNI103^{Cy5}) and dosed healthy mice with the labeled peptide by i.v., intraperitoneal (i.p.), and subcutaneous routes (s.c.). After 24 h, the animals were sacrificed and end-point tissue concentrations of CNI103^{Cy5} were determined by measuring the fluorescence intensities of the homogenized tissues and comparing to a standard line generated with known concentrations of the peptide. Following all three routes of administration, high levels of Cy5 fluorescence were detected in the liver, kidney, and spleen (Figure S12). Significant levels (>50 nM) were also present in other organs including the lungs, heart, pancreas, and lymph node, but not in the plasma or muscle. Based on these results, we conclude that CNI103 has good bioavailability after i.v., i.p., and s.c. administration, is broadly distributed into most organs and tissues (though less so into brain and muscle), and is still present in these organs and tissues at significant concentrations after 24 h. Analysis of splenic immune cell subsets following different routes of administration (i.p. and i.v.) revealed that CNI103 was rapidly and predominately internalized by myeloid lineage cells (e.g. neutrophils and monocytes) after all routes of administration, in addition to substantial uptake by unidentified non-T or B lymphocytes (Figure S13). The selectivity of CNI103 for myeloid lineage cells is likely because the latter generally have high rates of endocytosis and may make CNI103 advantageous as an anti-inflammatory agent. It should potentially inhibit macrophage/neutrophil-mediated inflammatory events but have less effect on adaptive immunity mediated by T and B lymphocytes. In sum, as an intracellular biologic, CNI103 has different PK properties from small-molecule drugs and the conventional plasma PK parameters are inadequate for predicting its *in vivo* effects. Instead, the half-life in tissue – especially target tissue (i.e., lung) – will be more relevant to the pharmacodynamic effects. Similar unconventional PK profiles have previously been observed for antisense oligonucleotides, which are another class of intracellular biologics.⁵⁵

CNI103 Prevents LPS-induced ALI in Mice.

To evaluate the *in vivo* efficacy of CNI103 for protection against LPS-induced ALI, CNI103 (3 mg/kg) was delivered by intranasal instillation in anesthetized mice. After 4 h, LPS (4 mg/kg) or saline was instilled to the nostrils of the mice. ALI parameters were analyzed after 6 h of LPS challenge. Intranasal LPS instillation in vehicle treated mice dramatically increased the levels of inflammatory cytokines (TNF α , IL-6) and protein in BALF as well as the lung wet to dry ratios (Figure 10). Pretreatment with CNI103 significantly reduced TNF α and IL-6 secretion, BALF protein levels and lung wet-to-dry ratios, compared to the vehicle control. BALF fluids were also collected at 24 h after LPS administration and the cytokine and protein levels were quantified. In both CNI103 and vehicle treated mice, the TNF α , IL-6, and protein levels had returned to the basal levels by 24 h (Figure S14). This indicates that CNI103 does not interfere with the natural repair processes of the lungs. To determine the lowest, effective dose of CNI103, we pretreated mice at three different concentrations, 3, 1, and 0.3 mg/kg (i.n.) for 2 h, challenged them with 10 mg/kg LPS (i.p.), and then measured the TNF α , IL-6, and protein levels in BALF. A clear dose response was observed, with the lower doses (e.g., 1 mg/kg i.n.) showing modest reduction in LPS-induced ALI parameters and less effective than the 3 mg/kg dose (Figure S15).

While intranasal administration of CNI103 is appropriate for ALI/ARDS and potentially other lung diseases, it may not be effective for inflammatory diseases in other organs/tissues. We therefore tested whether CNI103 is efficacious for ALI when administered by the i.v. route. Mice (n = 5) were pretreated with CNI103 at 5 mg/kg (i.v.) for 4 h and challenged with LPS (10 mg/kg i.p.). After 16 h, the ALI parameters were determined. The systemic efficacy of CNI103 was reflected by the dramatic reduction in the serum levels of TNF α and IL-6 relative to the vehicle control (DMSO; Figure 11A and 11B). CNI103 reduced BALF protein levels and the lung wet-to-dry ratios to the baseline values (Figure 11C and 11D). The effect of CNI103 on the BALF IL-6 and TNF α levels was less dramatic in this assay (Figure 11E and F), because by 16 h post LPS challenge the BALF cytokine levels had already returned to near baseline levels (supra infra). Finally, we collected the lungs and kidneys of the CNI103-treated and control mice and determined the TNF α levels in the tissue homogenates. While LPS increased the TNF α levels in the lungs of vehicle treated mice by ~30% (from 20 to 26 pg/mL), CNI103 (5 mg/kg i.v.) completely blocked LPS-induced TNF α secretion (Figure S16). As a control, there was no increase in TNF α secretion in the kidney upon LPS challenge, either in the presence or absence of CNI103. These findings demonstrate that CNI103 delivered by i.v. is systemically available and able to potentially reduce both lung and global inflammation.

Unique Properties and Potential Applications of CNI103.

As a novel CN inhibitor, CNI103 differs from CsA and FK506 in several key aspects. First, CNI103 is a more selective and less toxic than CsA and FK506. CNI103 exerts its biological activity by directly binding to CN, thus avoiding any toxicity associated with engaging a third-party protein such as cyclophilin and FKBP. Additionally, CNI103 specifically blocks the interaction between CN and a subset of protein substrates that contain the PxIxIT motif, but not all CN substrates, whereas the cyclophilin/CsA and FKBP/FK506 complexes sterically block the access of all protein substrates of CN.¹⁷ Second, CNI103 enters the cell by endocytosis followed by endosomal escape.^{41,49,50} This entry mechanism predicts that CNI103 should be more rapidly taken up by cells of high endocytosis and/or macropinocytosis rates. Our results indeed show that CNI103 enters myeloid lineage cells (e.g. neutrophils and monocytes) more efficiently than lung epithelial cells, T cells, and B cells. By contrast, CsA and FK506 enter cells by passive diffusion and are expected to enter a broad range of cell types more uniformly. This unique property renders CNI103 a highly effective inhibitor of the innate immune response, with potentially less effect on the adaptive immune response. The combination of an excellent safety profile and cell-type selectivity should make CNI103 an ideal candidate for treatment of a variety of inflammatory diseases. We have shown in this work that CNI103 is effective for preventing LPS-induced ALI/ARDS. We expect CNI103 to be effective for prevention and/or therapeutic treatment of ARDS caused by other mechanisms, including COVID-19 ARDS. We have also found CNI103 to be effective for treatment of inflammatory bowel diseases in a mouse model (data not shown). On the other hand, CNI103 lacked efficacy as an immunosuppressant for preventing transplant rejection in a mouse skin allograft model (data not shown), likely due to its inefficient entry into T cells.

CONCLUSION

Through a medicinal chemistry campaign, we developed a cell-permeable and metabolically stable peptidyl inhibitor, CNI103, which selectively blocks the interaction between calcineurin and NFATc3, thereby preventing the activation of NFATc3 *in vitro* and *in vivo*. In a mouse model of ALI/ARDS, local (i.n.) or systemic (i.v.) delivery of CNI103 (3–5 mg/kg) prevented the development of permeability pulmonary edema and lung inflammation in response to treatment with LPS. CNI103 represents the first chemical entity that specifically modulates the pathophysiology of ARDS with a desirable pharmacokinetic, pharmacodynamic, and toxicity profile. Our data indicate that CNI103 is a promising novel treatment for ARDS and, possibly, COVID-19 ARDS and other inflammatory diseases. This study also provides an important proof of principle that linear peptides can be developed into drug-like molecules to target challenging proteins such as those involved in intracellular protein-protein interactions.

EXPERIMENTAL SECTION

Materials.

Reagents for peptide synthesis were purchased from Advanced ChemTech (Louisville, KY) or Chem-Impex (Louisville, KY). 5-Fluorescein isothiocyanate (5-FITC) was purchased from Chem-Impex and 5(6)-carboxynaphthofluorescein, succinimidyl ester (NF-NHS) was from Molecular Probes (Eugene, OR). 2,3-Dichlorobenzoic acid, 4-bromo-3-chlorobenzoic acid, 2,3,4,5-tetrafluorobenzoic acid, 2,3-dichloroisonicotinic acid were purchased from Matrix Scientific (Elgin, SC). Solvents for synthesis and HPLC purification were purchased from Avantor Performance Materials (Paris, KY). Fetal bovine serum was purchased from VWR (Radnor, PA). Dulbecco's Modified Eagle Medium and Dulbecco's modified phosphate-buffered saline (DPBS) were purchased from Gibco (Gaithersburg, MD). Ninety-six- and 384-well microplates were purchased from Greiner Bio-One North America (Monroe, NC). Recombinant mouse M-CSF was purchased from PeproTech (Cranbury, NJ). TNF α and IL-6 ELISA kits were purchased from R&D Systems (Minneapolis, MN). RIPA buffer was from Cell Signaling Technology (Danvers, MA). Micro Bio-Spin columns were purchased from Bio-Rad (Hercules, CA). FaSSIF and FaSSGF powder were purchased from BioRelevant (London, United Kingdom). HeLa, HCT116, and H441 cells were purchased from ATCC (Manassas, VA). Jurkat-Lucia cells were purchased from Invivogen (San Diego, CA). Mouse hepatocytes were purchased from BioIVT (Westbury, NY). All other reagents and solvents and were purchased from Sigma-Aldrich (St. Louis, MO). All other disposable consumables were purchased from VWR. Antibodies for immunofluorescence and flow cytometry were purchased from the following suppliers: α -mouse CD45-PerCP/Cy5.5 and streptavidin-Alexa Fluor 594 from Biolegend (Cat. No. NC0217834 and 405240), α -rabbit IgG-Alexa Fluor 488 from Cell Signaling (Cat. No. 4412S), and Human/Mouse/Rat NFATc3 from R&D Systems (Cat. No. AF5834).

Wild type C57BL/6 mice were purchased from Jackson Laboratories and breeding colonies were maintained at The Ohio State University (OSU), Columbus. All of the animal experiments and procedures were conducted with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of OSU.

Protein Expression, Purification, and Labeling.

Calcineurin was expressed as a glutathione S-transferase fusion protein in *Escherichia coli* BL21 cells and purified on a glutathione-Sepharose column as previously mentioned.⁴⁰ For library screening, GST-CN was biotinylated by incubating 100 μ L of 16 mg/mL GST-CN (1.6 mg) in PBS (pH 7.4) containing 20% glycerol (v/v) with 2 eq. of NHS-biotin for 2 h at 4 °C. The reaction was quenched with 50 μ L of 1 M Tris-HCl, pH 8.0. Unreacted biotin-NHS was removed by passing the solution through a PD-10 desalting column and the material was used without further purification.

Molecular Dynamics.

MD was employed to provide clustered structures for docking using Schrodinger Glide.⁴⁶ Simulation was first prepared by editing the crystal structure of VIVIT/CN (pdbID: 2p6b) in UCSF Chimera⁵⁶ software suite to include ZIZIT structural modifications. All charges and protonation sites were calculated at pH 7.4. All system preparation and molecular dynamics were performed using GROMACS⁵⁷. The system was placed within a dodecahedral periodic box, each dimension 9.091 nm long, filled with copies of 216 equilibrated TIP3P water molecules and sufficient counter-ions for system neutralization. The system underwent two steps of energy minimization using steepest decent, first with only solvent relaxing, then the entire system followed by a heating process for which the temperature is increased from 0 to 300 K. The system then underwent two more phases of equilibration via MD, first using NVT (isothermal) conditions for 200 ps and then NPT (isothermal-isobaric with pressure control via the Parrinello-Rahman algorithm) conditions for 200 ps. The production MD simulations were performed under NPT conditions for 20 ns. Following the simulation, trajectory clustering was performed using *gmx cluster* using the *gromos* algorithm. Structures were clustered based on RMSD from the structure extracted from the 20-ns frame of the trajectory based on the RMSD of heavy atoms from CN residues F194, L270 and Y324 as these residues form the majority of the hydrophobic N-terminal proline-binding pocket. Snapshots every 4 ps from the trajectory were used for clustering, with the largest cluster representing ~90% of all structures sampled in the trajectory. A representative structure was produced for this cluster and served as the input receptor conformation for docking.

Docking.

Compounds for screening were constructed in Maestro and prepared for docking using LigPrep, with all ligands ionized to pH 7.4, all tautomers generated when applicable, and stereochemistry retained from the input structures. The receptor for docking was obtained from the clustered MD trajectory and was prepared using the Protein Preparation Wizard in Maestro to remove remaining water molecules and counter ions. The docking grid was generated using the Receptor Grid Generation tool in Maestro with no constraints input.

Docking was performed using Glide with XP precision with modified settings. The position of the C-terminal GP moiety in the original ligand was retained and used as a positional constraint for docking with a tolerance of 0.5 Å relative to the original ligand to reject poses not conforming to the constraint. Each compound was docked using flexible ligand docking, allowing nitrogen inversions and different ring conformations, but with the planarity of

conjugated π systems enhanced and nonplanar conformations of amides penalized. For conformer generation, enhanced sampling was used, and during initial pose selection, 200,000 poses per ligand were retained with the top 5000 poses per ligand kept for energy minimization combined with expanded sampling. Energy minimization was performed over 5000 steps and halogens were counted as hydrogen bond acceptors. Final docked compounds were ranked by their docking score and visualized using Maestro or UCSF Chimera.

Peptide Synthesis, Labeling, and Purification.

Peptides were synthesized on Rink Resin LS (0.48 mmol/g) using standard Fmoc chemistry. Synthesis of CNI103 and related compounds started with the linear CN-binding sequence, followed by the addition of the cyclic CPP (Scheme 1). Typical Fmoc deprotection was performed using 20% (v/v) piperidine in DMF twice for 5 min at RT with mixing. The typical coupling reaction contained 5 eq. of Fmoc-amino acid, 5 eq. of HATU, 5 eq. of HOBt, and 10 eq. of diisopropylethylamine (DIPEA) in 70:30 DMF/DCM (v/v) and was incubated for 1 h with mixing at RT. Fmoc-Hva-OH was similarly coupled without any side-chain protection. N-Terminal acyl groups were coupled using 5 eq. of the corresponding carboxylic acid, 5 eq. of PyBOP, 5 eq. of HOBt, and 10 eq. of DIPEA in 1:1 DMF/DCM (v/v) for 1 h with mixing at RT (twice). The Mtt protecting group on the C-terminal lysine was removed using 10 mL of 97:2:1 DCM/TFA/TIPS (v/v/v) for 5 min at RT (repeated for a total of 5 times) and synthesis was continued to prepare the CPP sequence using standard Fmoc chemistry. Finally, the allyl protecting group was removed from the C-terminal Glu by treatment with 0.3 eq. of *tetrakis*(triphenylphosphine)palladium(0) and 10 eq. of phenylsilane in dry DCM and in the dark for 15 min (repeated for a total of three times). The N-terminal Fmoc group was then removed with 20% piperidine in DMF. The resin was incubated in 1 M HOBt in DMF, washed with DMF and DCM, and the peptide was cyclized by using 5 eq. of PyBOP, 5 eq. of HOBt, and 10 eq. of DIPEA in 1:1 DMF/DCM for 30 min at RT (repeated once). For cleavage and deprotection of the peptide, the resin was treated with 1 mL of 92.5:2.5:2.5:2.5 TFA/TIPS/DMB/ddH₂O (v/v/v/v) per 50 mg of resin for 3 h at RT. All of the peptides were labeled on solid-phase (with protected side chains) using 1 eq. of 5-FITC or NF-NHS and 5 eq. of DIPEA in DMF overnight at RT. Crude peptide solutions were concentrated under a stream of inert gas and then triturated three times using chilled diethyl ether and the precipitate was isolated by centrifugation at 7.8K RCF, 4 °C. The supernatant was discarded and the crude peptide solid was dried under vacuum and stored at -20 °C until further purification. The crude peptide was dissolved in a minimal volume of DMF, diluted 1:10 with 1:1 ddH₂O/acetonitrile (containing 0.05% TFA) and purified by reversed-phase HPLC using a linear gradient of 5–60% acetonitrile in ddH₂O (containing 0.05% TFA) over 55 min. The HPLC was equipped with a Waters X-Bridge C18 semi-preparative column and a Waters 2457 UV/Vis detector monitoring 230 nm and 280 nm. All peptides were determined to have 95% or higher purity by analytical HPLC on a Waters X-Bridge C18 analytical column (monitored at 214 nm) and their authenticity was confirmed by HRMS (Figure S17).

Library Synthesis.

The library was synthesized on 1.0 g of TentaGel S NH₂ Resin (90 μm, 0.28 mmol/g). Synthesis began by coupling the common linker sequence, β-Ala-β-Ala-Arg-Arg-Met to the resin by following the peptide synthesis conditions described above (Figure S1A). Following N-terminal Fmoc removal, the resin (as a suspension in 28 mL of DMF) was volumetrically divided into 28 equal portions and placed into separate reaction vessels, to each of which a different Fmoc-amino acid (X⁴) was coupled (Figure S1B). The coupling reactions employed 4.5 eq. of Fmoc-amino acid, 0.25 eq. of CD₃CO₂H, 0.25 eq. of CH₃CD₂CO₂H, 5 eq. of HATU, 5 eq. of HOBt, and 10 eq. of DIPEA. The presence of the carboxylic acids resulted in chain termination for ~10% of the library peptides, facilitating later peptide sequence determination by MS.^{58,59} To synthesize the remaining random positions (X¹-X³), the resin from all 28 vessels was combined, mixed, and washed. Approximately 5 mg of the resin was set aside and the remaining resin (0.995 g) was treated with 20% piperidine in DMF to remove the N-terminal Fmoc group. The resin (0.995 g) was again split into 28 equal aliquots and to each aliquot a different Fmoc-amino acid was coupled, by using 4.5 eq. of Fmoc-amino acid, 5 eq. of HATU, and 5 eq. of HOBt. Additionally, 0.25 eq. of CH₃CD₂CO₂H + 0.25 eq. of CD₃CO₂H were added into the coupling reactions of Acp, βAsp, βAla, and Nle, whereas 0.25 eq. of CH₃CO₂H + 0.25 eq. of CD₃CO₂H were included into the coupling reactions of ala, leu, lys, and Orn. For all other amino acids, 0.5 eq. of CD₃CO₂H was added into their coupling reactions. The above split-and-pool procedure was repeated twice to synthesize the X² and X¹ positions. After all 4 random positions (X¹-X⁴) were added, the 4 groups of resin (3 × 5 mg + 0.985 g) were pooled, washed, and the N-terminal Fmoc group was removed and the peptide sequence Dcb-ZIZIT was coupled to all peptides by standard peptide chemistry. To increase the stringency of library screening and select for the most potent CN binders, the loading density of full-length peptides on the resin was reduced by 50%, by using 2.5 equiv of Fmoc-Thr(tBu)-OH, 1.25 eq. of CD₃CO₂H, 1.25 eq. of CH₃CD₂CO₂H, 5 eq. of HATU, 5 eq. of HOBt, and 10 eq. of DIPEA during the addition of the threonine residue (30 min). Sidechain deprotection was achieved by treating the resin with a modified reagent K (TFA/thioanisole/water/phenol/1,2-ethanedithiol, 82.5:5:5:5:2.5 v/v) for 3 h. The resulting library was exhaustively washed with DCM, DMF, 5% DIPEA in DMF, and 1:1 DCM/diethyl ether (v/v) and stored at -20 °C until use.

Library Screening.

A portion of the peptide library (200 mg) was placed in a 1.5-mL Micro-Spin column and washed extensively with MeOH, ddH₂O and PBS and blocked for 1 h with PBS containing 0.1% gelatin. Biotinylated GST-CN was diluted in 1 mL of blocking buffer (PBS, pH 7.4, 0.05% Tween-20, 0.1% gelatin [v/v]) supplemented with 5 mM TCEP to a final concentration of 50 nM and incubated on ice for 1 h. The resin was drained, resuspended in 1 mL of the GST-CN solution, and incubated overnight at 4 °C. The resin was gently drained and quickly resuspended (without washing) in 1 mL of SA-AP buffer (30 mM Tris, pH 7.6, 1 M NaCl, 10 mM MgCl₂, 70 μM ZnCl₂, and 20 mM K₂HPO₄) containing 1 μL of 1 mg/mL streptavidin-alkaline phosphatase. After incubation with mixing for 10 min at 4 °C, the resin was drained and washed with SA-AP buffer, PBS, and finally staining buffer (30 mM Tris, pH 8.5, 100 mM NaCl, 5 mM MgCl₂, and 20 μM ZnCl₂). The resin was transferred into a glass-bottomed petri dish by washing with staining buffer (total 1 mL final volume) and 80

μL of 5 mg/mL BCIP in staining buffer was added.⁵⁹ The mixture was incubated at RT on a rotary shaker and an intense turquoise color developed on positive beads in ~45 min, when the staining reaction was quenched by the addition of 1 mL of 1 M HCl. The positive beads (total 89 beads) were manually isolated with a micropipette under a dissecting microscope and separated into “intensely” (49 beads), “medium” (26 beads), and “lightly” colored categories (17 beads).

The hit beads were placed into individual 1-mL Eppendorf tubes (1 bead/tube) and the peptides were released from each bead by incubating the bead in 25 μL of 3 M CNBr dissolved in 70% TFA/H₂O (v/v) overnight at RT while protected from light. The peptide solution was dried under vacuum and dissolved in 10 μL of 1:1 acetonitrile/H₂O (v/v). One μL of the peptide solution was mixed with 1 μL of saturated α -cyano-4-hydroxycinnamic acid dissolved in 1:1 acetonitrile/H₂O and 1 μL of the mixture was spotted onto a Bruker 96-spot polished steel MALDI plate. MALDI spectra were recorded using a Bruker FLEX MALDI-TOF and analyzed using Bruker FlexAnalysis. A unique peptide sequence was inferred from the m/z ladder in each MS spectrum (Figure S2). The peptide sequences derived from the 49 intensely colored beads are listed in Table S2.

Fluorescence Polarization.

FP experiments were carried out in both direct binding and competition modes. For direct binding, 50 nM FITC-labeled peptide was prepared in PBS (pH 7.4) containing 5 mM DTT and 0.01% Triton-X100 and mixed with different concentrations of GST-CN solutions in PBS containing 0.01% Triton-X100. The solutions were incubated for 1 h at RT with gentle mixing and then transferred into black 384-well microplates. Fluorescence polarization was measured on a TECAN Infinite M1000 plate reader and titration curves were fitted using both KaleidaGraph v. 3.6 and GraphPad PRISM v.6 to the equation:

$$FP = \frac{\left(A_{min} + \left(A_{max} \times \frac{Q_b}{Q_f} - A_{min} \right) \left(\frac{(L + x + K_d) - \sqrt{((L + x + K_d)^2 - 4Lx)}}{2L} \right) \right)}{\left(1 + \left(\frac{Q_b}{Q_f} - 1 \right) \left(\frac{(L + x + K_d) - \sqrt{((L + x + K_d)^2 - 4Lx)}}{2L} \right) \right)}$$

where FP is the measured fluorescence polarization, A_{min} is the minimum FP value, A_{max} is the maximum FP signal, Q_b is the quantum yield of the bound fluorophore, Q_f is the quantum yield of the free fluorophore, L is the ligand concentration, K_D is the dissociation constant and x is the protein concentration.

For competition assays, 50 nM FITC-labeled probe was incubated with 50 nM GST-CN in PBS containing 5 mM DTT and 0.01% Triton-X100 for 1 h at RT with gentle mixing. Serial dilutions of competitor peptide were prepared in PBS plus 0.01% Triton-X100 and added to the above solution and equilibrated at RT with gentle mixing for 1 h. Samples were transferred into 384-well black microplates and FP values were measured on a TECAN Infinite M1000 plate reader and analyzed using GraphPad PRISM v.6 to determine IC_{50} values via a variable-slope four-parameter curve fit.

Solubility Determination.

Peptides were diluted from a DMSO stock into either PBS, pH 7.4 or PBS containing 5% DMSO (v/v) and then serially diluted in identical buffer to standardize DMSO concentration in clear, 96-well plates. The solutions were allowed to stand for 1 h at RT without mixing and turbidity was measured at 780 nm using a Tecan Infinite M1000 plate reader.

Serum Stability Assay.

Diluted human serum (25%; H4522 human serum, Sigma) was centrifuged at 15,000 rpm for 10 min, and the supernatant was collected. A peptide stock solution was diluted into the supernatant to give a final peptide concentration of 5 μ M and incubated at 37 °C. At various time points (0–24 h), 200 μ L aliquots were withdrawn and mixed with 100 μ L of 15% trichloroacetic acid and stored at 4 °C overnight. The final mixture was centrifuged at 15,000 rpm for 10 min in a microcentrifuge, and the supernatant was analyzed by reversed-phase HPLC equipped with a Waters XBridge C18 analytical column using a linear gradient from 5–60% acetonitrile (containing 0.05% TFA) in ddH₂O (containing 0.05% TFA). The amount of remaining peptide (%) was determined by integrating the area underneath the peptide peak (monitored at 214 nm) and comparing with that from the peptide at time 0.

Stability in Simulated Gastric and Intestinal Fluids.

Stability of CNI103 in simulated gastric fluid (SGF) was evaluated at 5 μ M CNI103 in FaSSGF buffer (pH 1.2), which was prepared with FaSSGF powder (#FFF01, BioRelevant). Similarly, stability of CNI103 in simulated intestinal fluid (SIF) was evaluated at 5 μ M CNI103 in FaSSIF buffer (pH 6.5), which was prepared with FaSSIF powder (#FFF-1017-B, BioRelevant) supplemented with lecithin and maleic acid. The solution was incubated at 37 °C for 5, 15, 30, 60, or 120 min, and the amount of remaining CNI103 was determined by LC/MS/MS analysis.

Intracellular Stability by Imaging Mass Spectrometry.

HCT116 cells were grown in McCoy's 5A media supplemented with 1% L-glutamine and 10% fetal bovine serum. Cells were cultured in 5% CO₂ at 37 °C. HCT116 spheroids were seeded with 7,000 cells in agarose-coated plates and grown for 12 days with half volume media changes every 2 days. They were prepared for imaging mass spectrometry analysis as previously described.⁶⁰ CNI103 was added to HCT116 spheroids to a final concentration of 5 μ M. After specified incubation times, media was aspirated and the spheroids were washed with 1x PBS. They were then embedded in gelatin. The spheroids were harvested and sectioned into 16 μ m-thick slices by using the gelatin-assisted sectioning method previously described.⁶⁰ Sections were thaw-mounted on ITO coated slides for MALDI MSI analysis. A TM sprayer nebulizer (HTX Technologies, Carrboro, NC) was used to apply the matrix for MALDI MSI analysis. The matrix, 2,5-dihydroxybenzoic acid (DHB), was dissolved in 50:50 acetonitrile/water with 0.1% TFA (EMB, Billerica, MA) to yield a final concentration of 10 mg/mL. Matrix was applied at 75 °C for 8 passes over the sample. The flow rate of matrix was 0.1 mL/min at a velocity of 1000 mm/min, track spacing of 2 mm, pressure 10 psi, gas flow rate of 3 L/min, nozzle height 40 mm, and a drying time of 20 s between each

pass. After matrix was applied, the sample was dried in a desiccator before MALDI MSI analysis.

An UltrafleXtreme MALDI-TOF-TOF spectrometer (Bruker Daltonics, Billerica, MA) was operated in positive ion mode with the reflector on and set to acquire a mass range of 360–1400 m/z. Laser raster was set to 40 μ m along both the x and y axes. Each MALDI mass spectrum for each pixel is a result of 200 consecutive laser shots. External calibration was performed using a standard peptide mixture on one spheroid. The images were processed using FlexAnalysis 3.0 and FlexImaging 4.1 (Bruker Daltonics, Billerica, MA). All spectra were normalized against total ion count, defined as the sum of all intensities in the mass range analyzed to reduce influences by matrix hot spots. All samples were analyzed in biological triplicate and technical triplicate. Heatmaps, scaled relative to the most intense signal, were generated for each time point and fragment (Figure S11).

Hepatocyte Stability Assay.

Mouse hepatocytes (#M005052, BioIVT) were revived following manufacture's protocol with cryopreserved hepatocyte recovery medium and used for the assay when cell viability was greater than 70%. Testing compound was mixed with mouse hepatocytes to give a final peptide concentration of 2 μ M and a final hepatocyte cell density of 2 million per mL. The cells were incubated at 37 °C in a humidified incubator in the presence of 5% CO₂. At varying time points (0, 15, 30, 60, and 90 min), 30- μ L aliquots of the reaction mixture were withdrawn for LC-MS/MS based quantification. The reaction (30 μ L) was quenched with 200 μ L of 1:1 (v/v) methanol/acetonitrile containing 25 ng/mL terfenadine as the internal standard. After vigorous mixing (by vortex) and centrifugation, 100 μ L of the supernatant was diluted two-fold with distilled water and analyzed by reversed-phase HPLC coupled with a Sciex 4500 Q-trap mass spectrometer.

Liver Microsome Stability Assay.

Mouse liver microsome was obtained from XenoTech (Cat. No. M1000). A stock solution of CNI103 (10 mM) was prepared in DMSO and diluted to 100 μ M in a phosphate buffer (50 mM potassium phosphate, pH 7.4). The CNI103 solution was gently mixed with the liver microsome to give a final concentration of 0.5 mg/mL and incubated at 37 °C. At varying time points (0, 5, 15, 30, and 60 min), 25- μ L aliquots of the reaction mixture were withdrawn for LC-MS/MS based quantification. The testing solution (25 μ L) was quenched with 300 μ L of 1:1 (v/v) methanol/acetonitrile containing 25 ng/mL terfenadine as the internal standard, followed by vigorous mixing (by vortex) and centrifugation. The supernatant (100 μ L) was diluted two-fold with distilled water and analyzed by reversed-phase HPLC coupled to a Sciex 4500 Q-trap mass spectrometer. To evaluate metabolism by P450 enzymes, the microsome degradation reaction was similarly carried out, except that the reaction was supplemented with a NADPH solution (final concentration 1 mM).

Ex Vivo TNF α /IL-6 Quantification in Mouse Lung Macrophages.

Bone marrow cells from wild type C57BL/6 mice were isolated and allowed to differentiate into mature bone marrow derived macrophages (BMDMs) as previously described.⁶¹ BMDM were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with

10% FBS and 1% penicillin/streptomycin. Differentiated lung macrophages were pretreated with CPP9-VAVAA, CsA, or CNI93/CNI103 at indicated concentrations and stimulated with LPS (100 ng/mL for 8 h) or PBS. TNF α or IL-6 secretion into the culture medium was determined using R&D Systems Quantikine ELISA kits.

Cellular Uptake Assay.

HeLa cells maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin were seeded into 12-well plates (1.5×10^5 cells per well) and incubated overnight at 37 °C, 5% CO₂. The following day, the cells were washed with DPBS and incubated with 5 μ M NF-labeled peptide dissolved in DMEM containing 10% FBS, 1% penicillin/streptomycin for 2 h at 37 °C in the presence of 5% CO₂. After that, the cells were washed with DPBS and harvested from the plate by treatment with 0.25% trypsin for 5 min. Cells were centrifuged at 300x RCF, washed twice with cold DPBS, and resuspended in cold DPBS for flow cytometry analysis on a BD LSR II flow cytometer followed by data analysis and gating using Flowjo.

Cell Viability Assays.

HeLa, Jurkat-Lucia, or H441 cells were seeded into a 96-well microplate (5000 cells per well) in full growth media and incubated at 37 °C overnight. A serial dilution of test compound was prepared in DPBS and added to each well. The treated cells were incubated at 37 °C with 5% CO₂ for 72 h. Following compound treatment, 10 μ L of MTT stock solution was added to each well. After an additional 4 h at 37 °C, 100 μ L of SDS-HCl solubilizing solution was added to each well and the plate was returned to the incubator overnight at 37 °C. A Tecan Infinite M1000 Pro microplate reader was used the following morning to measure the absorbance of the formazan product at 565 nm and viability was calculated relative to untreated control cells.

Mouse lung macrophages were plated in a white opaque 96-well plate (Greiner Bio-one, Cat # 655083) at a density of 10,000 cells per well in 90 μ L of RPMI media supplemented with 1% penicillin/streptomycin and 10% fetal calf serum. A serial dilution of CNI103 was prepared in DPBS and 10 μ L of peptide solution was added to each well. DMSO concentration was kept constant at 0.5% (v/v) in all wells and the cells were incubated at 37 °C with 5% CO₂ for 16 h. Following compound treatment, 100 μ L of CellTiter-Glo® 2.0 cell viability assay reagent (Promega, Cat #G9242) was added into each well and the plate was incubated with shaking for 15 min at room temperature. Luminescence from each well was measured on a Tecan M1000 Pro plate reader.

CN Phosphatase Activity.

p-Nitrophenyl phosphate (50 mM) was dissolved in assay buffer (100 mM Tris, pH 7.5, 100 mM NaCl, 1 mM MnCl₂, 0.4 mM CaCl₂, 0.1 mg/mL BSA, 1 mM DTT) in a quartz cuvette. GST-CN was incubated with CNI103 or DMSO for 30 min at 4 °C before being added into the cuvette to give final concentrations of 50 nM for GST-CN and 50 μ M for CNI103. Hydrolysis of pNPP was continuously monitored at 405 nm and the phosphatase activity was determined from the slope of the early regions of the reaction progress curve (60 s).

Pulse Oximetry.

Following peptide delivery after 4 h, the fur around the neck of each mouse was shaved and respiratory rate of each mouse was measured using MouseOx Plus Pulse Oximeter (Starr Life Sciences Corp, USA) in accordance with manufacturer's instructions. Each mouse was allowed to acclimatize for 30 min after placement of a collar clip sensor. Breaths/min were recorded for 5 min for 5 times and then averaged.

Immunofluorescence.

Mouse BMDM were grown on glass bottom cell imaging dishes and then pretreated with 1 μ M CPP9-VAVAA-biotin or CNI93-biotin for 1 h and stimulated with LPS (100 ng/mL) or PBS for 1 h. Treated cells were washed, fixed in 4% paraformaldehyde and incubated sequentially with rabbit NFATc3, α -rabbit Alexa Fluor® 488 conjugate and Alexa Fluor® 594 streptavidin. Cells were washed three times with PBS after incubation with each antibody. Localization of NFATc3 (Alexa Fluor® 488) and peptide (CPP9-VAVAA or CNI93 in Alexa Fluor® 594) with PBS/LPS stimulation was determined using Nikon A1R microscope (Nikon, Inc.) equipped with 488 nm and 543 nm excitation lasers.

In Vivo Uptake and Distribution in Lung Cells.

TMR-labeled CNI93 (5 mg/kg) or 10% DMSO in saline was delivered intranasally into anesthetized mice. After 1, 2, 4, 16 and 24 h, mice were euthanized and total lung cells were isolated by collagenase/DNase digestion to obtain single cell suspension as described previously.¹⁴ Total lung cells were stained with PerCP/Cy5.5 α -mouse CD45, CD31, CD326 antibodies and analyzed by flow cytometry.

Mouse Model of LPS-Induced Acute Lung Injury.

Peptide (0–10 mg/kg) or an equal volume of DMSO (vehicle) was delivered either intranasally in 25 μ L of 1% DMSO in sterile saline (v/v) or by i.v. (5 mg/kg), in 0.5% DMSO in sterile saline (v/v) to anesthetized mice and after 2–6 h, treated with LPS (i.p. 10 mg/kg or i.n. 4 mg/kg). After 16 h (i.p.) or 4 h (i.n.) post LPS administration mice were euthanized using ketamine/xylazine and acute lung inflammation was measured in terms of increased BALF levels of protein, IL-6, TNF α , and plasma levels of IL-6 and TNF α . In addition, neutrophilic infiltration into BALF was determined by Hema 3.0 staining and number of neutrophils in BALF was quantitated.

Maximum Tolerated Dose.

Peptides were delivered into anesthetized mice and monitored for gross changes in body weight and body scores over a period of 9 or 10 days. Changes in body weights of mice administered with 10 mg/kg CPP9-VAVAA i.n., 10 mg/kg CNI93 i.n. and 50 mg/kg CNI93 i.p. were measured.

In Vivo PK Studies of CNI103.

All animal studies were conducted and approved under protocol BD-201807188. BALB/c male mice (6-week old, ~25 g) were obtained from LingChang and acclimated for 1 week before start of the study. The animals were housed in rooms maintained between 20–26 °C

and 30–70% relative humidity and on a 12-h light/dark cycle with food and water available ad libitum. Mice were intravenously administered CNI103 (5 mg/kg) dissolved in sterile PBS to a volume of 5 mL/kg. At each various time points (5 min, 15 min, 30 min, and 2 h, 4 h, and 8 h), approximately 30 μ L of blood was collected with K₂EDTA as anticoagulant. Whole blood was centrifuged, and plasma was separated and collected for storage at –80 °C until processing and analysis. Linear range of quantification by LC-MS/MS of CNI103 was established from 5–1000 nM. The concentration of CNI103 was determined by LC/MS/MS analysis.

Biodistribution.

CNI103^{Cy5} was administered to anesthetized mice via three routes (i.v., i.p., and s.c.) with or without human serum albumin (HSA) added as an excipient. Plasma samples were obtained as designated time points through retro-orbital bleeding. End-point tissue peptide concentration was determined following homogenization by fluorescent intensity and standardized to a fluorescent peptide signal of known concentration spiked into each tissue matrix.

For characterization of CNI103^{Cy5} uptake in splenic immune cells, harvested cells from each plasma collection time point were stained with anti-CD45, CD3, B220, and Mar-1 antibodies and analyzed by flow cytometry. Cell types were differentiated as follows: neutrophils (high SSC and Mar-1⁺), monocytes (high SSC and Mar-1⁺), T cells (low FSC and SSC and CD3⁺), B cells (low FSC and SSC and CD3-B220⁺). Among the low FSC and SSC, all other subsets of immune cells were identified as CD45⁺ CD3[–] B220[–] as CD45 is a marker of bone marrow derived white blood cells. There was a population of CD3⁺ B220⁺ cells, but this was likely artefactual antibody binding as this combination is not possible. After each population was identified, the percentage of Cy5⁺ cells and the mean fluorescence intensity (MFI) of Cy5 of each population were then determined and plotted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ALI	acute lung injury
ARDS	acute respiratory distress syndrome
BALF	bronchoalveolar lavage fluid

Chg	L-cyclohexylglycine
cisPro	2,2-dimethyl-1,3-thiazolidine-4-carboxylic acid
CN	calcineurin
CPP	cell-penetrating peptide
CsA	cyclosporine A
Dcb	2,3-dichlorobenzoyl
Den	2,3-dichloroisonicotinyl
FITC	fluorescein isothiocyanate
FP	fluorescence polarization
Hva	β -hydroxyvaline
IL-6	interleukine-6
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intravenous
LPS	lipopolysaccharide
NF	naphthofluorescein
NFAT	nuclear factor of activated T cells
PPI	protein-protein interaction
Tle	<i>tert</i> -leucine
TNFα	tumor necrosis factor α
VIVIT	GPHPVIVITGPHEE
ZIZIT	GPHPZIZITGPHEE

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Figure 1. Compound optimization strategy. (A) Binding mode of peptide ZIZIT-cisPro to CN (PDB ID: 2P6B) as derived from MD simulations. (B) Structure of peptide ZIZIT-cisPro and the N- and C-terminal regions modified during optimization are shown in red and blue colors, respectively. Figure 1A is reproduced from Ref 40. Copyright 2020 American Chemical Society.

Figure 2.

A docking model showing the binding interaction between Dcb-ZIZITGP and CN (PDB ID: 2P6B). The peptide ligand is shown as sticks, whereas the ligand-binding surface of CN is shown in salmon. Peptide atoms are shown in yellow (carbon), blue (nitrogen), red (oxygen), and green colors (chlorine).

Figure 3.

Histogram showing the sequence selectivity of CN C-terminal to the ZIZIT motif. Data shown are number of occurrence (y axis) of a particular amino acid (x axis) at a given peptide position X^{1-4} (z axis). Structures for each amino acid monomer are located in Fig. S1.

Figure 4:

Biochemical properties of calcineurin inhibitor CNI93. (A) Structure of CNI93. (B) Binding affinity of CNI93 to CN as determined by a FP-based competition assay. (C) Comparison of the serum stability of VIVIT, ZIZIT-cisPro, and CNI93. (D) Cytosolic entry efficiency of NF-labeled CNI93 into HeLa cells (after treatment with 5 μ M peptide for 2 h) as determined by flow cytometry analysis. Data shown are relative to that of CPP9 (100%).

Figure 5:

Effect of CNI93 on NFATc3 nuclear translocation and cytokine secretion ex vivo. **(A,B)** Mouse bone marrow-derived macrophages were pretreated with 1 μM biotinylated CPP9-VAVAA **(A)** or CNI93 **(B)** and stimulated with LPS (100 ng/mL) or PBS for 1 h. Intracellular NFATc3 (green) and biotinylated peptide (red) were visualized using Nikon A1R microscope equipped with 488 nm and 543 nm excitation lasers. Single confocal optical sections (pinhole set to achieve 1 Airy unit) are shown (n = 7–10 cells/group). Scale bars, 10 μm. **(C,D)** Inhibition of LPS-induced TNFα and IL-6 secretion by CNI93. Total lung macrophages from healthy mice were pretreated for 4 h with indicated concentrations of DMSO, CPP9-VAVAA, CsA, or CNI93 and stimulated with LPS (100 ng/mL for 8 h) or PBS. Statistical significance determined by one-way ANOVA to vehicle (0.1% DMSO) treated cells, *, p < 0.05, **, p < 0.01, ***, p < 0.001.

Figure 6: Time-dependent distribution of CNI93^{TMR} in mouse lung tissues following intranasal administration (5 mg/kg; n = 4). (A) TMR fluorescence in BALF. (B) TMR fluorescence in leukocytes (all CD45⁺ cells) and CD31⁺/CD45⁻ epithelial cells. Values are given as average and S.D. of n = 4 animals.

Figure 7.

CNI93 attenuates LPS-induced ALI in mice. Anesthetized mice were dosed (i.n. 10 mg/kg) with CNI93 or CPP9-VAVAA (VAVAA) and after 2 h injected with LPS (i.p. 10 mg/kg). After 16 h, BALF levels of IL-6 (A) and TNF α (B), and plasma levels of IL-6 (D) and TNF α (E), as well as BALF protein (C), lung wet/dry ratio (F) were determined. (G) A differential count of macrophages and neutrophils was determined on a cytospin slide with Hema 3.0 and multiplied by the total number of cells in the BALF to determine the total number of macrophages and neutrophils in each sample. Data shown represent the mean $\pm$ SEM from five mice in each group. Statistical difference between different treatments was calculated by one-way ANOVA with Bonferroni correction. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 8.

In vitro/Ex vivo properties of CNI103. (A) Binding of FITC-labeled CNI103 to CN as analyzed by FP. (B) Serum stability of CNI103 and Tat at 37 °C. Data shown represent the percentage of intact peptide remaining as a function of incubation time and are relative to that at time zero (100%). (C and D) Effect of CN inhibitors on LPS-induced production of TNF α and IL-6 by mouse lung macrophages. Data were analyzed by one-way ANOVA relative to vehicle (0.1% DMSO) treated cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Note that data in (C), (D), and Figure 5C and 5D were derived from the same experiment and share the same controls (DMSO, CPP9-VAVAA, and CsA).

Figure 9.

In vivo PK profile of CNI103 in mice following i.v. injection (5 mg/kg). (A) Plot of plasma concentration as a function of time. The two data points at 2 and 4 h are arbitrarily placed just below 5 nM, which is the detection limit of the LC/MS/MS method. (B) CNI103 concentrations in major organs 8 h after i.v. injection. The error bars represent the mean $\pm$ SD from four mice in each group.

Figure 10.

Protection against LPS-induced ALI in mice by intranasally administered CNI103 (3 mg/kg). (A) IL-6 in BALF; (B) TNFα in BALF; (C) total protein levels in BALF; and (D) lung wet-to-dry ratios 6 h after LPS challenge. Error bars represent the mean ± SEM from five mice in each group. Statistical difference between different treatments was calculated by one-way ANOVA with Bonferroni correction. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 11.

Protection against LPS-induced ALI in mice by systemically administered CNI103 (5 mg/kg). (A) IL-6 in plasma; (B) TNFα in plasma; (C) total protein in BALF; (D) lung wet to dry ratios; (E) IL-6 in BALF; and (F) TNFα in BALF 16 h after LPS challenge. Error bars represent the mean ± SEM from five mice in each group. Statistical difference between different treatments was calculated by one-way ANOVA with Bonferroni correction. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Scheme 1.**Solid-Phase Synthesis of CNI103**

Reagents and conditions: a) standard SPPS; b) 20% piperidine in DMF; c) 2,3-dichloroisotryptophan, PyBOP, HOBT (5 equiv); d) 2% TFA and 1% triisopropylsilane in DCM; e) Fmoc-Glu(OH)-OAll, HATU, HOBT (5 equiv); f) Pd(PPh₃)₄ (0.3 equiv), PPh₃ (10 equiv); g) 1 M HOBT in DMF; h) PyBOP, HOBT; and i) TFA.

Table 1.

N-Terminally Acylated ZIZIT Peptides and Their Predicted Binding Energies and Experimental CN-Binding Affinities

Peptide	Acyl Group	Docking Score (kcal/mol)	K_D (nM)
1	Ac-GPHP		92 ± 12
2		-12.8	224 ± 56
3		-11.6	357 ± 22
4		-12.1	207 ± 40
5		-10.0	>5400
6		-9.5	>5800
7		-12.8	32 ± 12

Peptide	Acyl Group	Docking Score (kcal/mol)	K_D (nM)
8		-12.9	74 ± 15
9		-13.4	41 ± 9
10		-12.9	189 ± 10
11		-13.3	77 ± 27

Table 2.

CN-Binding Affinity of Peptides Selected from the C-terminal Peptide Library

Peptide	Sequence ^a	K _D (nM) ^b
12	Dcb-ZIZIT-miniPEG-Lys(FITC)	700 ± 150
13	Dcb-ZIZIT-Tyr-glu-Fpa-His-βAla-Lys(FITC)	12 ± 4
14	Dcb-ZIZIT-Gln-His-leu-Ser-βAla-Lys(FITC)	9.1 ± 3.9
15	Dcb-ZIZIT-Gln-His-leu-His-βAla-Lys(FITC)	4.7 ± 1.4
16	Dcb-ZIZIT-Asp-Gln-Tyr-Arg-βAla-Lys(FITC)	64 ± 31
17	Dcb-ZIZIT-Asp-Gln-Tyr-lys-βAla-Lys(FITC)	22 ± 3

^aD-Amino acids are shown in all-lowercase letters. Dcb, 2,3-dichlorobenzoyl; βAla, β-alanine, FITC, fluorescein isothiocyanate; Fpa, 4-fluorophenylalanine.

^bData represent the mean ± SD of three or more independent experiments.

Table 3.

Sequences and CN binding affinities of CNI95 derivatives.

ID	Sequence	IC ₅₀ (nM) ^a
21 (CNI95)	Dcb-Tle-Ile-Tle-Ile-Thr-Gln-His-leu-Ser-miniPEG-Lys-CPP9	150 ± 29
22 (CNI101)	Dcn -Tle-Ile-Tle-Ile-Thr-Gln-His-leu-Ser-miniPEG-Lys-CPP9	180 ± 10
23	Dcb- Hva -Ile-Tle-Ile-Thr-Gln-His-leu-Ser-miniPEG-Lys-CPP9	ND
24 (CNI102)	Dcb-Tle-Ile- Hva -Ile-Thr-Gln-His-leu-Ser-miniPEG-Lys-CPP9	ND
25 (CNI103)	Dcn -Tle-Ile- Hva -Ile-Thr-Gln-His-leu-Ser-miniPEG-Lys-CPP9	220 ± 10
26	Dcn -Tle- Chg - Hva -Ile-Thr-Gln-His-leu-Ser-miniPEG-Lys-CPP9	45 ± 1
27	Dcb-Tle-Ile-Tle-Ile-Thr-Gln-His-leu- Arg -miniPEG-Lys-CPP9	190 ± 60
28	Dcb-Tle-Ile-Tle-Ile-Thr-Gln-His- val -Ser-miniPEG-Lys-CPP9	250 ± 35
29	Dcb-Tle-Ile-Tle-Ile-Thr- Asn -His-leu-Ser-miniPEG-Lys-CPP9	290 ± 5
30	Dcb-Tle-Ile-Tle-Ile-Thr- Asp -His-leu-Ser-miniPEG-Lys-CPP9	1520 ± 50

^aND, not determined.