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Covalent modification of nephrilin peptide with valproic acid increases its efficacy as a therapeutic in burn trauma

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Abstract

Introduction: Nephrilin peptide, a designed inhibitor of Rictor complex, modulates systemic responses to trauma, alleviating clinically relevant variables in a rat scald model and sepsis mortality in a mouse model. This study explores the possibility that chemical conjugation of small molecules to the aminoterminus of nephrilin can modify its biological activity in the rat scald model.

Methods: One of four molecules (valproic acid, decanoic acid, fenofibric acid and ibuprofen) was chemically attached to the amino terminus of nephrilin during synthesis. Animals were treated with each modified nephrilin by subcutaneous bolus injection on days 1–7 post-burn.

Results: Compared to nephrilin, valproic acid-modified nephrilin showed significantly (all $p < 0.05$) improved systemic effects on kidney function (creatinine 0.17 ± 0.03 vs 0.31 ± 0.09 mg/dL), glycemic control (AUC 57.5 ± 40 vs 136.4 ± 69.2 mg.dL.hr), inflammation (IL-6 24 ± 9 vs 39 ± 8 pg/ml), pathological angiogenesis (1.46 ± 0.87 vs 6.53 ± 3.16 pct pixels) and weight gain (3.74 ± 0.31 vs 2.99 ± 0.53 slope), all variables previously shown to bear upon clinically relevant burn injury outcomes.

Conclusion: Modification of nephrilin with valproic acid increases the efficacy of nephrilin peptide in burns.

Keywords

Nephrilin; Burn injury; Valproic acid; Immune modulation; Kidney function; Glycemic control; Pathological angiogenesis

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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1. Introduction

The peptide nephrilin is a designed inhibitor of Rictor complex derived by fusing a 19 amino acid segment of Protor with the metal binding domain (MBD) of human insulin-like growth factor binding protein-3 (IGFBP3) — a 21 amino acid sequence that targets and preferentially enters stressed cells when injected subcutaneously into rodents, and specifies homeostatic immunomodulatory functions. The mechanism (which is not fully understood) may involve interactions with nuclear receptors [1-3]. Severe burn trauma is associated with a vast array of secondary systemic effects including hyperinflammation, hypercatabolism, sepsis, organ failure, loss of glycemic control, delayed wound healing and cognitive deficits. These serious and enduring complications can lead to substantial morbidity and mortality [4-9]. We previously demonstrated the efficacy of nephrilin peptide in combating many of the pleotropic effects of burns [10-12]. Nephrilin treatment following burn injury reverses epigenetic and signaling changes in kidney tissue that lead to the activation of Rac1, and lowers elevations in markers of systemic oxidative stress such as urinary 8-isoprostane and plasma OHDG. In a recent publication we describe the variables most reliably modulated by nephrilin in the rat scald model, these variables falling into in seven distinct efficacy categories [12,13]. In addition, an analysis of gene expression in the central nervous system after burn injury showed that nephrilin beneficially modulated the expression of genes associated with astrocytosis, oxidative stress and immunosuppression [14].

Nephrilin has previously been shown to modulate the neuroimmune response to a variety of xenobiotic and metabolic stressors in rodents [1,10,15]. When injected into mice at high doses daily for 26 days, nephrilin generates no visibly differential pathology compared to vehicle [1]. The nephrilin MBD is known to bind ferrous ($Fe2^{+}$) and ferric ($Fe3^{+}$) iron. Based on crosslinking studies, uptake of this metal-binding domain into mammalian cells involves binding to integrin-beta-3, a component of a major metal uptake pathway, and to transferrin receptor [2,3,16]. Iron increases the efficacy of nephrilin in the rat scald model [12].

In this study we explore the impact of a seven-day treatment regimen comprising subcutaneous bolus injection of ironsupplemented nephrilin peptide with or without chemical modification. Modification of peptides by covalent addition of small molecules at the aminoterminus has been widely employed in research. For example, biotin is often added as a handle for subsequent detection, and dyes can be added for histological visualization. However, the systematic screening of covalently modified immunomodulatory peptides using, as adducts, molecules that are immunomodulatory drugs themselves, is less common. In order to streamline translational application of this general approach, we have focused of small molecules that have a free carboxylic group, in order to facilitate conjugation as a final step in conventional peptide synthesis. In this study we have used valproic, decanoic and fenofibric acids as well as ibuprofen, as chemical adducts to nephrilin peptide. Valproic acid, decanoic acid and ibuprofen were selected for their known anti-inflammatory effects, and fenofibric acid for its effects on glycemic control [17-20]. Valproic acid, an HDAC inhibitor, is a particularly interesting adduct to explore in the context of immodulin peptides like nephrilin, whose mechanism of action is still unclear, but is believed to also involve physical interactions with nuclear receptors (manuscript in preparation), as has previously

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been shown for the parent molecule from which the sequence of nephrilin is derived, IGFBP-3, referenced above.

2. Materials and Methods

2.1. Reagents

Nephrilin peptides amino-terminally conjugated to valproic acid (N1vlp), decanoic acid (N1dec), fenofibric acid (N1fen) or ibuprofen (N1ibu) were synthesized by Kinexus Bioinformatics Corporation (Vancouver, BC) and purified to > 70% by HPLC. The design and synthesis of the nephrilin peptide (N1) and the benefits of iron supplementation have been previously described [1,12]. Antibodies for ELISAs were purchased from Abcam (Cambridge, MA), and chemicals from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.2. Peptide administration

Adult male Sprague Dawley rats (250–300 gm, Charles River Laboratories, Wilmington, MA, USA) were injected with nephrilin peptides plus equimolar ferric iron once daily by subcutaneous bolus injection, days 1–7 post-scald. The first dose was administered after completion of the scald procedure. Injection volume was 400 uL. Control animals received the same volume of vehicle. Treatment group sizes were (n = 6) unless otherwise indicated: group S = sham-treated; group B = burn + vehicle; group N1 = bu rn + 2 mg/kg nephrilin; group N1vlp = burn + 2 mg/kg N1vlp peptide; group N1dec = burn + 2 mg/kg N1dec peptide; group N1fen = burn + 2 mg/kg N1fen peptide; group N1ibu = burn + 2 mg/kg N1ibu peptide. A 4 mg/kg daily dosage of nephrilin previously demonstrated efficacy in eleven different rodent disease models [1,10-15]. Moreover, in a safety study, mice that were treated daily with 20 mg/kg nephrilin by subcutaneous bolus for 26 days showed no differential toxicology in major organs when compared to a saline control [1]. We chose a lower dose (2 mg/kg) for this experiment to make potential improvements in the efficacy of the modified peptides more obvious.

2.2.1. Rat scald model—The rat scald burn model [21] is a modified Walker-Mason model that induces inflammation and hypermetabolism in line with what severely burned patients experience. The model results in a mortality rate of <1%. Adult male Sprague Dawley rats were housed in clean cages on a 12 hr light/dark cycle with access to food (standard chow) and water *ad libitum.* Animals were allowed to acclimate for one week prior to the experiment. All animal procedures were performed in adherence to the National Institute of Health's *Guide for Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee (IACUC) of the Molecular Medicine Research Institute. All procedures were initiated in the morning between 7 and 10 a.m. Prophylactic analgesia (0.05 mg/kg body weight Buprenorphin) was administered 15 min before general anesthesia using isofluorane. The dorsum of the trunk and the abdomen were shaved, and a 60% of total body surface area (TBSA) burn administered by placing the animals in a mold and immersing them in 98–100 °C water for 10 s on the back and 2 s on the abdomen, except that for anatomical reasons female rats received only the dorsal burn, thereby reducing the burn exposure for female rats. This method delivers a full-thickness

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cutaneous burn as confirmed by histological examination. Burned rats were immediately resuscitated with 40 ml/kg body weight Ringer's Lactate injected intraperitoneally, as in the original referenced study [21]. Animals in the sham group were treated exactly as described above for burned animals except that the animals were placed in room temperature water. Animals were randomly assigned to treatment groups, and nephrilin peptides or vehicle were administered by subcutaneous bolus daily. At the end of the study period animals were euthanized by decapitation as approved by MMRI IACUC guidelines, the NIH's Office of Laboratory Animal Welfare (OLAW), and AVMA recommendations. All tissues and organs of interest were rapidly dissected or collected and flash frozen in liquid nitrogen for subsequent storage at −80 °C.

2.3. Glucose tolerance test

Fourteen days post-burn, the rat tail was snipped and the baseline glucose level measured using a BAYER contour blood glucose monitoring system. The rats were injected intraperitoneally with glucose and readings were performed at 30 min, 60 min, and 120 min post-injection. Results were expressed as AUCs (μg/dL/hr) over baseline over the sampling period.

2.4. Early hyperinflammation and inflammasome activation

24 h after scald, a blood sample was taken from each (isofluorane anesthetized) rat. Plasma IL-6 was measured using a rat IL-6 DuoSet ELISA kit (R&D Systems, Minneapolis, MN). Blood taken at 14 days post-burn was analyzed for 27 cytokine and chemokine analytes including VEGF-A, IL-18, IL1-beta, CCL5 and CXCL5 (RD27 Custom Plex Discovery Assay, Eve Technologies, Calgary, AB).

2.5. Plasma OHDG

14-day plasma was assayed for OHDG using an Oxidative Damage High Sensitivity ELISA Kit purchased from Cayman Chemical (Ann Arbor, MI).

2.6. Kidney function (plasma creatinine)

Kidney function was indirectly assessed by measuring 14-day plasma creatinine. eGFR (estimated glomerular filtration rate expressed as ml/min/100 g animal body weight) can be computed from this value as previously described [11,22].

2.7. PctRedPix computation

A digital image of each wound at 4 weeks post-scald were analyzed using GIMP 2.10 software. Red pixels, as a percentage of all pixels within the wound area were counted by the software and expressed as a percentage of total.

2.8. Computation of efficacy

Aggregate efficacy of each treatment regimen was computed using an average of Z-scores calculated from the distribution of values for each analyte by subtracting the mean of the distribution from the value and dividing by the standard deviation for the distribution.

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2.9. Statistical analysis

Data are presented as means ± standard deviation (SD) unless otherwise indicated. Probability values (*p* values) were computed using Student's *t*-test and expressed relative to sham or salinetreated group.

3. Results

3.1. Chemical modification with valproic acid improves the efficacy of nephrilin peptide

Fig. 1 shows the results obtained when male rats in the scald model were exposed to vehicle, 2 mg/kg nephrilin or nephrilin peptide modified with valproic acid, decanoic acid, fenofibric acid or ibuprofen. The dose of peptide used in this study is half the dose traditionally used to show the efficacy of nephrilin peptide in this rat model. The results demonstrate the improved effectiveness of valproic-modified nephrilin peptide (N1vlp) in numerous readouts believed to be harbingers of clinically relevant outcomes in burn injury. Plasma levels of IL-6 at 24 h post-insult have shown a correlation to mortality and morbidity in critical illness. At 2 mg/kg, N1vlp reduces 24-hour elevation in IL-6 in this model significantly, whereas the same dose of the parent molecule nephrilin does not. At day 14, elevations in plasma IL-18 and IL1-beta are diagnostic for inflammasome activation, which is a marker of chronic inflammation in stress models [23]. All nephrilins appeared to be effective in reducing IL1-beta, but IL-18 is significantly reduced only by N1vlp and N1ibu in this study.

Although angiogenesis is necessary for wound healing, excessive angiogenesis delays healing [24]. In a recent study, we showed beneficial effects of nephrilin peptide on four readouts of pathological angiogenesis: VEGF-A, CCL5, CXCL5 and a visual measure of healing, PctRedPix, measurement of which is described in the Methods section. [12]. In the current study, all nephrilins tested were effective at reducing these readouts of excessive angiogenesis significantly.

Systemic oxidative stress (as indicated by plasma OHDG levels) is another marker relevant to wound healing [12]. In this study, both nephrilin and N1vlp significantly reduced plasma levels of OHDG.

Three particularly important and clinically relevant measurements shown in Fig. 1 are the glucose-tolerance test (GTT), plasma creatinine (as a surrogate for kidney function), and weight loss (as a proxy for lean mass). The beneficial effects of nephrilin treatment on each of these readouts has been documented in previous studies [11,12]. In the current study, the superiority of N1vlp over the parent molecule and other modified nephrilins is clearly visible for each of these three measures.

To make global comparisons of treatment efficacy for the various treatment groups, we converted the values obtained from all samples in each assay to z-scores (by subtracting the mean of all values and dividing the result by the standard deviation of the distribution). This assigned each treatment group a z-score for each assay. We next averaged all assay z-scores for a treatment group within each efficacy class, creating an unweighted aggregate z-score for each efficacy class. For example, the "angiogenesis" class used an unweighted average of four assay readouts: VEGF-A, CCL5, CXCL5 and PctRedPix. Finally, a composite

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"Effectiveness Score" was an unweighted mean of aggregated z-scores from all seven effect classes. Effectiveness Score allows direct comparison of efficacy across treatment groups. The value for sham (0.82) is the positive control. The negative control is the Burn + vehicle group (−0.61). For convenient visual representation, values were placed on a 1–100 scale between these two controls (Burn + vehicle = 1, Sham = 100). Fig. 2 shows a plot of such values for peptides and controls.

4. Discussion

Dysregulated host immune responses to traumatic stress, massive infection and other severe challenges often exhibit a timecourse of hyperinflammation followed by immunosuppression, catabolism syndrome and chronic critical illness [25]. This immunological phenomenon represents an alarming and rapidly expanding burden on the healthcare system. Nephrilin's efficacy in reversing the systemic effects of sepsis and burn trauma, including the loss of glycemic control, body mass, kidney function, wound healing capacity and sepsis [11,12] appears to involve a unique pro-homeostatic immune modulation of this broad dysfunction. In other rodent models of stress, nephrilin peptide also ameliorates elevations in inflammatory and oxidative stress consequent to metabolic and xenobiotic insult [1,15]. In burn injury models, readouts such as elevated plasma IL-6 at 24 h post-insult, chronic inflammasome activation, loss of body mass, wound healing impaired by excessive angiogenesis, loss of glycemic control, elevated markers of oxidative stress, immune dysregulation and neurodegenerative consequences, among others, have emerged as emblematic of the underlying phenomenon's progression [25-27].

In this study, we show that covalent linkage of nephrilin peptide to the small molecule valproic acid more than doubles the efficacy of the peptide in the model. Although valproic acid has been used to treat seizures for decades, the underlying mechanisms of action for this molecule are poorly understood. Antiinflammatory and epigenetic effects are commonly mentioned, but exactly how these actions are mediated is not well established [18].

This study confirmed each of our earlier findings regarding the robust effects of nephrilin peptide on early IL-6 elevation, and other clinically relevant effects on glycemic control, kidney function, inflammasome activation, pathological angiogenesis, loss of body weight and systemic oxidative stress in the rat scald model [10-12]. This study showed that valproic acid-modified nephrilin peptide was superior to the parent molecule at addressing each of these phenomena, thereby supporting our conjecture that these effects may be linked at a systemic mechanistic level. The translational impact of this study lies in the creation of a substantially improved drug candidate, N1vlp peptide.

Although nephrilin studies have involved collaborations between our group and six other academic research laboratories, it is important to note that no data have been reported for nephrilin independently of us and these collaborations.

Our results raise an obvious question for future study: Can this peptide be coupled to other small molecules to improve even further upon its effectiveness in burn trauma? We intend to address this question in future experiments.

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Abbreviations:

Rac1	Ras-related C3 botulinum toxin substrate 1
OHDG	8-Oxo-2′-deoxyguanosine
ELISA	enzyme-linked immunosorbent assay
MMRI	Molecular Medicine Research Institute
NIH	National Institutes of Health
AVMA	American Veterinary Medicine Association
AUC	Area under the curve
IL-6	interleukin-6
VEGF-A	vascular endothelial growth factor-A
IL-18	interleukin-18
IL1-beta	interleukin-1-beta
CCL5	chemokine (C–C motif) ligand 5
CXCL5	C-X-C motif chemokine 5
eGFR	estimated glomerular filtration rate
GTT	glucose tolerance test

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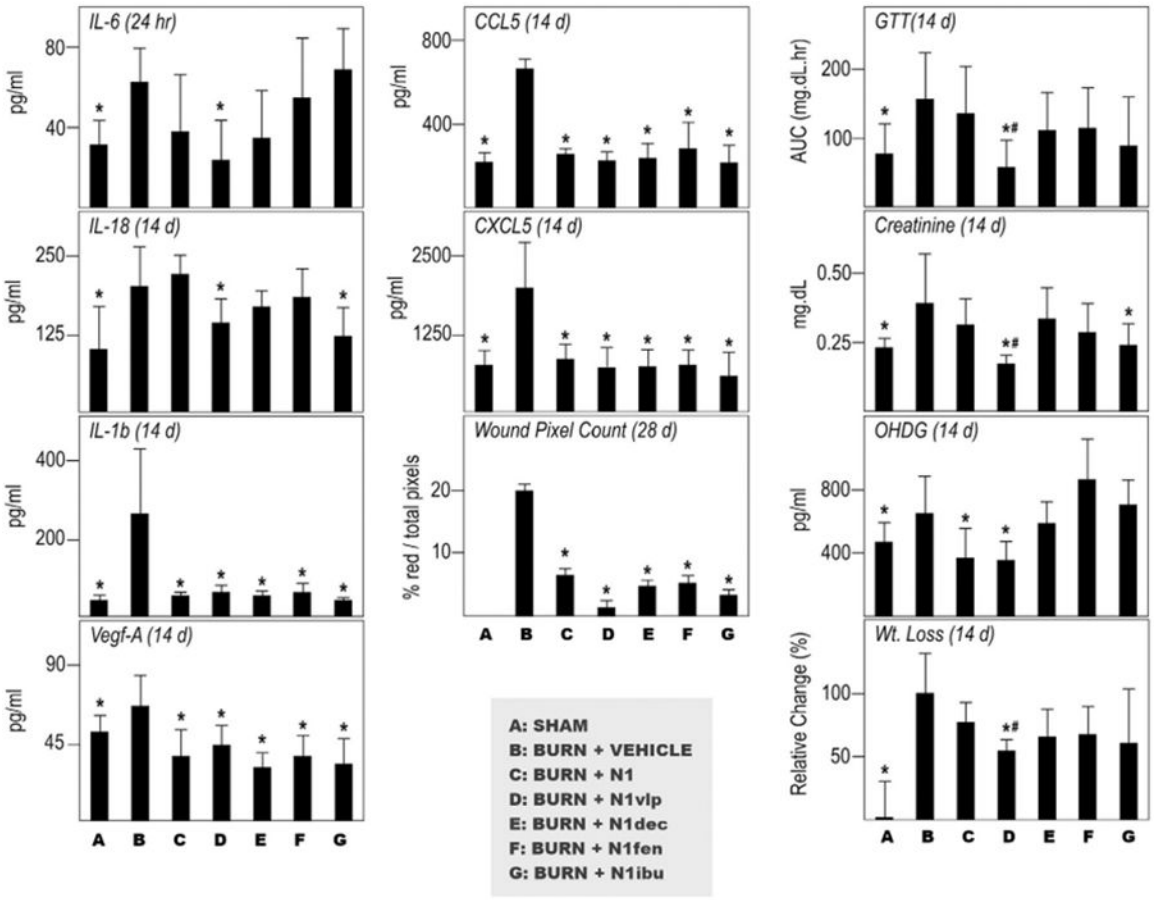

Fig. 1.

Efficacy of modified peptides in the rat scald model. See text and Methods for a description of the assays. * $p < 0.05$ vs B group; # $p < 0.05$ vs C group.

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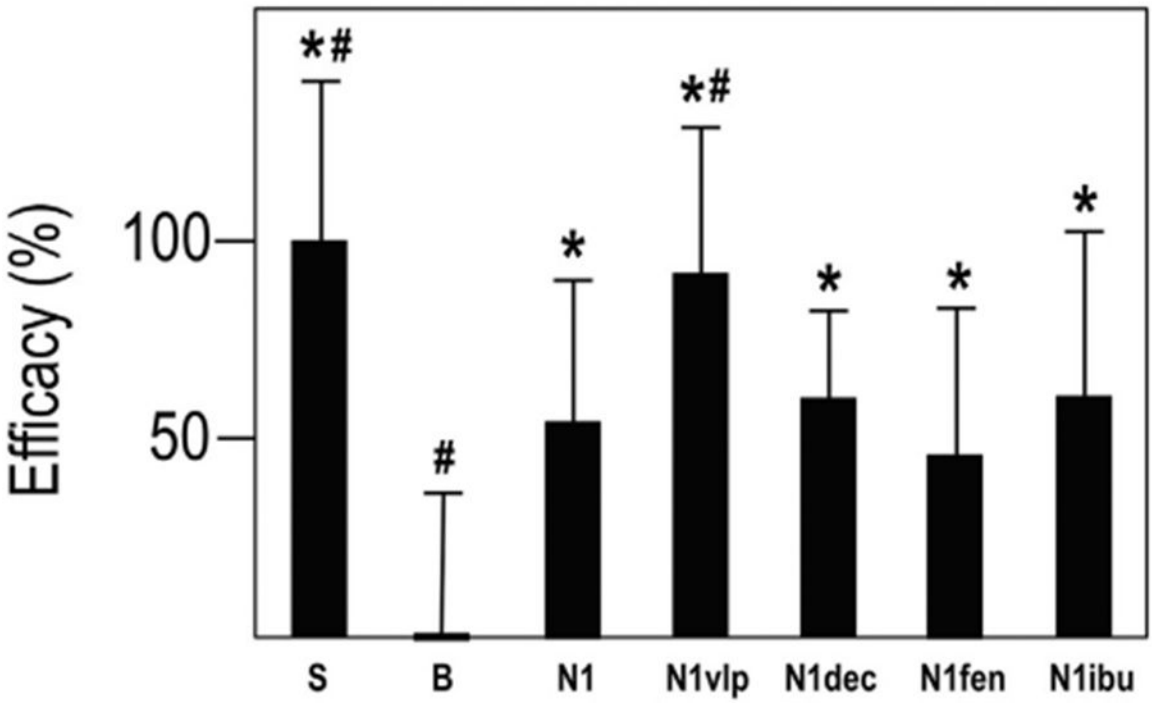

Fig. 2.

Standardized aggregate efficacy plot for the seven treatment groups, with sham group set to 100 and vehicle group set to 1 for each sex. See text. *p < 0.05 vs B group; # p < 0.05 vs N1 group.