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NMR and molecular modelling studies on elastase inhibitor-peptides for wound management

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ABSTRACT

Proteases play an important and critical role in the physiological process of wound repair. However, excessive and unregulated release of proteolytic enzymes (e.g., elastase) mediates abnormal degradation of healthy tissues, which leads to inflammatory disorders such as chronic wounds. Thus, it is of therapeutic interest to develop novel synthetic inhibitor-peptides of elastase, which can restore the balance between the free enzyme and the endogenous inhibitors in chronic wounds. In previous works, we have reported two different drug delivery systems to release novel elastase inhibitors to the wound site. In both systems synthetic peptides (KRCCPD*T*CGIKCL-Pep4 and KRMMPD*T*MGIKML-Pep4M) based on the primary structure of the endogenous elastase inhibitor, secretory leucocyte protease inhibitor, were used as active material. Phosphorylation of the reported peptides prompts significant structural differences, which reflects in distinct inhibitory capacity towards elastase. These structural modifications were prompted by electrostatic interactions and hydrogen bonds established from the peptide phosphoresidue. The current study was also extended to another synthetic peptide (WC*TAS*VPPQCY-PepBBI) that is based on the reactive loop of another elastase inhibitor, the Bowmen-Birk inhibitor. PepBBI, phosphorylated and non-phosphorylated, displays similar behaviour to Pep4 and Pep4M. The structural modifications reported herein were evaluated by two-dimensional nuclear magnetic resonance and molecular modelling approaches.

1. Introduction

Normal wound healing is a controlled balance between repair processes that lead to new tissue formation and destructive processes, which are essential to remove damaged tissue [1,2]. The perfect control of the biological processes involved in healing leads to normal wound repair. However, any alteration in these physiological processes may lead to a non-healing state and the formation of a chronic wound [1]. Chronic wounds fail to progress through the normal stages of healing and therefore enter a state of pathologic inflammation [2,3]. The prolonged inflammatory phase characteristic of chronic wounds results in exuberant response of neutrophils [4]. Neutrophils invade the inflammatory sites and their massive accumulation at the inflammation site mediates the release of high amounts of proteases (concentrations reaching millimolar range) and free radical generating enzymes that become deleterious to the healing process [4–7]. The proteolytic environment of the wound resulting from high neutrophil concentrations is a combination of cationic serine proteases (e.g., human neutrophil elastase (HNE)) and matrix metalloproteinases (e.g. collagenase MMP-8 and gelatinase MMP-9) [4,6]. These two families of proteases, when at normal levels, augment wound healing. However, at high levels are associated with the degradation of important growth factors and major proteins of the extracellular matrix necessary for wound healing. Among the proteases released from neutrophils, human neutrophil elastase (HNE) is capable of cleaving tissue proteins such as elastin, which plays a major function in the arteries, skin and ligaments, collagen (Types I–V, IX and XI), fibrin, fibronectin, cartilage, proteoglycans, and cytokines [5,8,9]. Elastase action is modulated by multiple endogenous inhibitors, such as α1-protease inhibitor, secretory leucocyte protease inhibitor (SLPI) and elafin. Still, under pathological conditions the oxidative inactivation of these natural protease inhibitors takes

Abbreviations: MMPs, matrix metalloproteinases; PPE, porcine pancreatic elastase; SLPI, secretory leukocyte protease inhibitor; Pep4, KRCCPDTCGIKCL; Pep4(P), Pep4 phosphorylated; Pep4M, KRMMPDTMGIKML; Pep4M(P), Pep4M phosphorylated; PepBBI (Bowman-Birk inhibitor), WCTASVPPQCY; PepBBI(P), PepBBI phosphorylated; Thr(P), Phosphothreonine; 2D NMR, two-dimensional nuclear magnetic resonance; COSY, 2D correlated spectroscopy; TOCSY, 2D total correlated spectroscopy; NOESY, nuclear Overhauser enhancement and exchange spectroscopy; NOE, nuclear Overhauser enhancement or nuclear Overhauser effect; RMSD, root mean square deviation.

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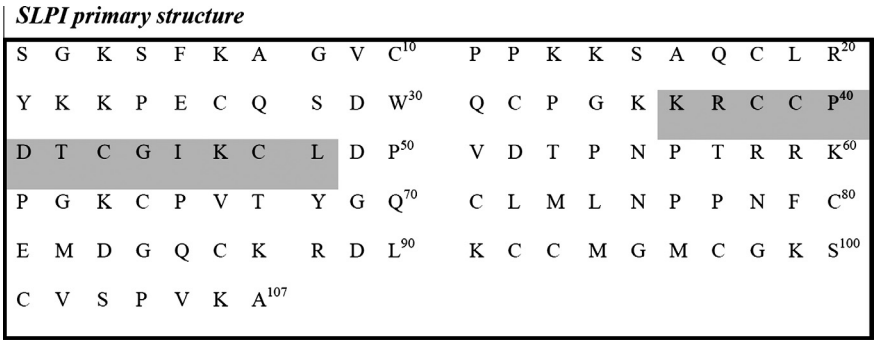

Fig. 1. Secretory leukocyte protease inhibitor (SLPI) primary structure. Identification of Pep4 sequence in SLPI structure (in grey).

place [10–13], leading to a protease-antiprotease imbalance. Under chronic inflammation, the imbalance is in favour of proteases, which widely overwhelm the inhibitory capacity of the endogenous proteinaceous inhibitors [5]. To reverse this protease-antiprotease imbalance and reinforce the elastase inhibitor's activity, different types of inhibitors and inhibitor formulations have been developed for wound dressings therapeutic applications. The design of synthetic low molecular weight, peptide-based [14–16] or non-peptide-based [17–19], elastase inhibitors has received considerable attention due to their potential therapeutic usefulness. The delivery of inhibitors or other active materials to the wound medium, promoted by wound dressings, can be accomplished by two different approaches: (i) the sequestration of elastase directly from the wound environment, with further protease inactivation or (ii) the direct release of the protease inhibitor into the wound medium, to further control the elastase levels in chronic non-healing wounds [4]. In previous studies [20,21], we have reported two different drug delivery systems, based on both wound dressing mechanisms described herein, to deliver novel elastase peptidic inhibitors to wound environment. The peptide sequence of these inhibitors, KRCCPDTCGIKCL (Pep4) and KRMMPDTMGIKML (Pep4M) [20], was selected from an endogenous elastase inhibitor, secretory leucocyte protease inhibitor (SLPI, Fig. 1). Pep4M is a mutation of Pep4 sequence, having replaced all cysteine residues by methionine residues. Both peptides revealed differential inhibitory activity towards elastase (porcine pancreatic elastase, PPE) when a specific residue (threonine, in this case) is in the phosphorylated and non-phosphorylated form [20]. The structure of peptides Pep4 and Pep4M and the influence of this posttranslational modification on their structure were evaluated herein by two-dimensional NMR and molecular modelling studies. Additionally, these studies were also extended to another elastase inhibitor-peptide, PepBBI (WCTASVPPQCY), a synthetic peptide derived from the Bowman-Birk Inhibitor reactive loop [14,22–24], in order to establish a comparison between PepBBI and the previous peptides. Similarly to Pep4 and Pep4M, for PepBBI a distinct inhibitory behaviour was also observed relatively to elastase for the phosphorylated and non-phosphorylated version of the peptide (unpublished data), which is reflected in the structural conformations adopted by the peptide in solution.

2. Materials and methods

2.1. NMR experiments

2.1.1. NMR sample preparation

The elastase inhibitor-peptides Pep4 (KRCCPDTCGIKCL), Pep4M (KRMMPDTMGIKML) and PepBBI (WCTASVPPQCY) used in this study were custom-made by JPT Peptide Technologies GmbH (Berlin, DE) [25].

The NMR analysis of the tridecameric peptides (Pep4 and Pep4M) [20] and undecameric peptide (PepBBI) [14,24] was performed in aqueous solution (90% H_2O/10% ^2H_2O) at 283 K to slow down molecular tumbling and at pH 3.0 to minimise the rate of exchange of NH protons with the solvent. Working at pH below 7 is standard for structural determination of proteins by NMR. The NMR samples were prepared at a concentration of approximately 4 mg/mL using water (with 10% D_2O) as solvent, for signal lock.

2.1.2. NMR spectra acquisition and analysis

NMR data were acquired on a Bruker Avance II+ spectrometer operating at proton frequency of 500 MHz (Fig. 2). Two-dimensional homonuclear (2D) NMR experiments were performed: a ^{1}H-Double-quantum filtered COSY [26] with 2k points covering a spectral width of 6 kHz and 512 increments in the indirect dimension; a TOCSY [27] with 60 ms of spin-lock time and a 2k points covering a spectral width of 6 kHz and 512 increments in the indirect dimension and a NOESY [28,29] with 300 ms mixing time and 2k points covering a spectral width of 6 kHz and 1k increments in the indirect dimension. NOE buildup experiments were performed to confirm that with this mixing time the condition of initial buildup is still valid. In all cases suppression of the solvent signal was obtained using a Watergate sequence with gradients. Additional NOESY experiments were performed using 300 ms of mixing time in a Bruker Avance III NMR spectrometer, operating at 800 MHz using a QXI probehead. 2k points covering a spectral width of 10.4 kHz were measured for 1k increments in the indirect dimension, to obtain further structural constrains. The solvent signal was suppressed using excitation sculpting with gradients. The Topspin software was used to process the data. All experiments were zero filled to duplicate the number of points in both dimensions. NOESY and TOCSY data were multiplied in both dimensions by a cosine squared windowing function prior to Fourier transform. COSY data were multiplied in both dimensions by a sine squared function prior to Fourier transform. Baseline correction was made following Fourier transformation using a 5th degree polynomial function. Peak lists of the three aforementioned 2D spectra were generated by interactive peak picking with further automatic integration of the peak volumes in the SPARKY software [30] (Table SI1–3). Peak assignments in COSY, TOCSY and NOESY spectra were performed using the sequential assignment method described by Wüthrich [31,32]. The chemical shifts and volumes of the NOESY crosspeaks were converted into XEASY format and input in the CYANA 2.0 program for structure calculations and analysis.

2.1.3. Structure determination

Assignments of ambiguous NOESY cross peaks were obtained by using the module CANDID in the program CYANA [33]. A total of 100 conformers were generated by CYANA and the 20 conformers with lowest target function were used to represent the 3D NMR structural bundle (Table 1). The quality of the structures was