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Cyclization enhances function of linear anti-arthritic peptides

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Abstract This study describes the biophysical and immunomodulatory features of a cyclic peptide termed C1 which consists of alternating D-, L-amino acids and is capable of inhibiting IL-2 production *in vitro* and reducing the induction and extent of T-cell mediated inflammation in animal models. Solid-state nuclear magnetic resonance demonstrates that the peptide orders the lipid bilayer, suggesting a transmembrane orientation, and this is supported by surface plasmon resonance indicating strong binding affinity of C1 to model membranes. *In vitro* cell viability and proliferation assays show that C1 does not disrupt the integrity of cell surface membranes. Permeation studies of C1 and analogs across human epidermis cells show that the stability and skin permeability are enhanced by cyclization. Treatment with C1 in an asthma and in an arthritis animal model resulted in a suppressed immune response. Cyclization may be a useful means of enhancing biological linear peptide activity and improving delivery.

1. Introduction

There is an increasing awareness and appreciation of the use of peptides in human disease [1,2]. Linear peptides have limitations and some of these problems may be overcome by cyclization. Molecules in nature are often brought to bioactive conformation by ring formation. One such example is the cyclic peptide derived from α-fetoprotein reported to inhibit estrogen-stimulated growth of immature mouse uterus and estrogen-dependent proliferation of human breast cancer cells [3]. Another example of a cyclic peptide produced naturally by fermentation is cyclosporin, an immunosuppressant drug that

Abbreviations: AA, amino acid; ACD, At-Column Dilution; BALF, bronchoalveolar lavage fluid; C1, cyclic peptide 1; C1-L, linear sequence of C1; CP, core peptide; DIEA, N,N-diisopropylethylamine; DMF, dimethylformamide; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol; DMSO, dimethylsulfoxide; FDPP, pentafluorophenyl diphenylphosphinate; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IL, interleukin; MOPS, 3-(N-morpholino) propane sulfonic acid; MTB, *Mycobacterium tuberculosis*; NMR, nuclear magnetic resonance; PBL, peripheral blood lymphocyte; PG, propylene glycol; PMA, Phorbol 12-myristate 13-acetate (PMA); SEA, staphylococcal enterotoxin A; SPR, surface plasmon resonance; TCR, T cell antigen receptor; SUV, small unilamellar vesicles; TFA, trifluoroacetic acid.

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is widely used to prevent or delay rejection of solid organ allografts or xenografts in skin, heart, kidney, pancreas and lungs [4]. In addition to naturally occurring cyclic peptides, synthetic cyclic peptides with various modifications have been shown to have biological activity [5]. Hartgerink et al. [5] and Fernandez-Lopez et al. [6] have shown that six and eight residue cyclic D, L-α-peptides act on gram positive and gram negative bacterial membranes causing rapid cell death by increasing permeability and collapsing transmembrane ion potentials. Cyclic peptides with an even number of D- and L-amino acids adopt a flat, ring-shaped conformation with the amide functional groups oriented perpendicular to the side chain allowing the cyclic peptides to stack and form hollow like tubular structures (nanotubes) [5]. The effectiveness of this class of peptides as selective antibacterial agents is highlighted by Fletcher et al. [7] who reported the discovery of six-residue cyclic D, L-α-peptides with diverse activity towards two prokaryotic bacteria (gram-positive methicillin-resistant *Staphylococcus aureus* and gram-negative *Escherichia coli*) and two eukaryotic marine algae, *Ulva linza* and *Navicula perminuta*. These peptides have the potential to be used for the development of new biofouling agents that are not generally toxic to all organisms but rather specifically target microbial agents of interest.

Recently Monteiro et al. [8] reported a family of amphiphilic eight residue D, L-α-cyclic peptides that specifically blocked entry of all tested HCV genotypes into target cells at a post binding step without affecting infection by other enveloped RNA viruses. Motiei et al. [9] further developed glycosylated membrane active self-assembling cyclic D, L-α-peptides and showed that these peptides can significantly reduce mammalian cell toxicity while maintaining potent bactericidal activities against multidrug-resistant gram-positive species. In essence, cyclic D, L-α-peptides are proteolytically stable, easy to synthesize, and can be derived from a vast number of potentially membrane-active sequences. The unique abiotic structure of the cyclic peptides and their rapid bactericidal action may also limit temporal acquirement by drug resistant bacteria. The lower molecular weight D, L-α-peptides offer an attractive complement to the current arsenal of naturally derived antibiotics, and hold considerable potential in combating a variety of existing and emerging infectious diseases.

We have previously investigated a linear peptide (GLRILLLKV; CP) and shown it to exhibit immunomodulatory activity [10]. CP consists of nine amino acids, the sequence of which has been derived from the T-cell antigen receptor (TCR)-α chain transmembrane region, and CP is able to inhibit IL-2 production in T-cells following antigen recognition. Extending these studies *in vivo*, CP, given subcutaneously or intraperitoneally, significantly reduced the induction of T-cell mediated inflammation in animal models with adjuvant induced arthritis, allergic encephalomyelitis and delayed type contact hypersensitivity [11,12]. However, there are limitations in delivering and stabilizing linear peptides to the desired site in an intact and biologically active form. To overcome these problems we designed and synthesized a number of D-, L-cyclic peptides and examined their stability and biological activity. Here we report the biological and biophysical properties of one cyclic peptide termed C1 and show that there is improvement of function and augmentation of physico-chemical characteristics compared with the linear peptide.

2. Materials and methods

2.1. Peptide synthesis

Peptides were synthesized by standard solid phase peptide synthesis methods, using side-chain protected Fmoc-conjugated amino acids in the manual mode [13]. Fmoc-protected amino acids were purchased from Auspep (Melbourne; Australia) or Novabiochem (Sydney; Australia). The linear sequences were constructed using tritylchloride polystyrene resin (Novabiochem) loaded with the first protected amino acid. The addition of subsequent amino acids was achieved by first deprotecting the Fmoc group followed by coupling of the next amino acid. The cycle was repeated until the last amino acid was coupled, then the Fmoc group was removed with 50% piperidine in dimethylformamide (DMF) and the linear protected peptide was cleaved from the resin with 1% trifluoroacetic acid (TFA) in dichloromethane and collected using pyridine/methanol (3/7) mixture. The linear peptide was then cyclized either in DMF (~1 mM solution) with PyBOP (Novabiochem; 4 Eq), N,N-diisopropylethylamine (DIEA; 10 Eq) or by using cyclizing reagent FDPP as reported by Skropeta et al. [14]. In the latter method cyclization was achieved under anhydrous conditions, using 1.5 Eq of pentafluorophenyl diphenylphosphinate (FDPP; 39 mg, 0.1 mmol) with a solution of linear protected C1 (100 mg, 0.007 mmol) in acetonitrile (14 mL), followed by addition of 3 Eq of DIEA (0.2 mmol). The reaction mixture was then stirred at room temperature overnight, after which the solvent was removed by rotary evaporation prior to characterization by mass spectrometry. Deprotection was achieved with standard TFA/scavengers mixture. Large scale C1 was purchased from Peptide International (USA) at a >90% purity.

2.2. Peptide purification

Purification of C1 was achieved by using At-Column Dilution (ACD) technique developed by Waters. This technique was developed for injecting relatively large volumes of strong sample diluents. ACD technique also prevents bulk precipitation in the sample loop or in the column itself. Briefly, C1 (100 mg) was dissolved in approximately 0.5 mL of acetonitrile, 200 μL of 10% TFA and then DMSO was added until a clear solution was obtained. The sample was centrifuged prior to collecting the supernatant for loading on to a C18 reverse phase column. Sample was loaded using ACD techniques. C1 was loaded on to the column at 0.1 mL/min flow rate. Simultaneously mobile phase was pumped through the column using another pump at a flow rate of 5 mL/min. Presence of C1 in fraction collected at a retention time of 31.8–32.2 min was confirmed by mass spectrometry.

2.3. Antigen presentation assay

This is an established assay [10] used to assess T cell activation by measuring IL-2 produced in response to antigen. Briefly, 2B4.11 hybridoma (5×10^4 cells) and LK35.2 (5×10^4) were incubated with pigeon cytochrome C (50 μM) in 96-well microtiter plates for 24 h in the presence and absence of peptides (50, 25 and 10 μM, respectively). An aliquot of each