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Short Communication

ENHANCED PHAGOCYTOSIS ACTIVITY OF CYCLIC ANALOGS OF TUFTSIN

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Abstract—Cyclic analogs of the physiological immunostimulating peptide tuftsin (Thr-Lys-Pro-Arg), cyclo(Thr-Lys-Pro-Arg-Gly) (ctuf-G) and cyclo(Thr-Lys-Pro-Arg-Asp) (ctuf-D), were synthesized based on molecular modeling studies, and assayed for the ability to stimulate phagocytosis by human polymorphonuclear leukocytes. As predicted, the synthesis of ctuf-D resulted in two isomers with the correct molecular mass and amino acid composition. In phagocytosis assays, tuftsin, ctuf-G and two isomers of ctuf-D showed the usual bell-shaped activity profiles. The optimum concentration of ctuf-G was 50-fold less than that of tuftsin, whereas the degree of stimulation was similar. One isomer of ctuf-D was almost inactive, and the other ctuf-D exhibited the same degree of phagocytosis as tuftsin but its optimum concentration was 5-fold lower. The enhanced potency of ctuf-G and one isomer of ctuf-D may be due to conformational effects and/or to the possibility that these cyclic peptides are resistant to proteolytic degradation.

Key words: tuftsin; cyclic tuftsin analogs; phagocytosis

Tuftsin is a natural tetrapeptide (Thr-Lys-Pro-Arg) first noted by Najjar and Nishioka [1] to stimulate phagocytosis by PMN.† Tuftsin binds directly to specific tuftsin receptors on PMNs, monocyte-macrophages and NK cells, and modulates their biological activities [2, 3]. Tuftsin deficiency has been found in patients with some types of cancer, myelofibrosis, idiopathic thrombocytopenic purpura, splenectomy, sickle cell disease, AIDS and AIDS-related complex, in addition to tuftsin congenital abnormalities [2–5]. In animal and clinical studies, tuftsin has displayed antitumor, anti-infection, and anti-AIDS activities with no detectable toxicity [2, 3, 6].

Many attempts were made originally to produce active tuftsin analogs, as reviewed [7]. These efforts have been largely unsuccessful primarily due to either loss of activity or formation of competitive inhibitors, although these studies did serve to emphasize the specific structural requirements of the molecule. Although mice maintained on tuftsin dissolved in the *ad lib* water supply in our experiment demonstrated some antitumor activity [8], tuftsin is known to be degraded rapidly in serum with a half-life of several minutes [9, 10]. Therefore, peptidase-resistant tuftsin analogs would permit preparation of tablets for oral administration, thus facilitating clinical trials in cancer, sickle cell disease, and AIDS, which are now dependent on intravenous infusions. In addition, an ecto-enzyme on the membrane of PMNs, leucine aminopeptidase, inactivates tuftsin by cleaving the N-terminal threonine producing a competitive inhibitor, Lys-Pro-Arg [11]. As a result, tuftsin analogs that are resistant to this peptidase attack would be expected to possess enhanced therapeutic potential, and efforts in this direction have been reported by modifying the N-terminal side of the molecule [10, 12–15].

Cyclized peptides can be very resistant to peptidases as shown recently with peptide T [16]. Chipens *et al.* [17] previously synthesized a series of cyclic tuftsin analogs, which showed reduced *in vitro* phagocytosis activity compared with the parent molecule. To identify new cyclic tuftsin analogs in a more systematic manner, O'Conner *et al.* [18] carried out molecular modeling studies of linear tuftsin and of cyclic analogs using quenched molecular dynamics techniques in both aqueous and DMSO environments. The effects of cyclization and overall molecular charge on backbone conformation were studied by modeling ctuf, ctuf-G, ctuf-D, and ctuf-K. Of these four cyclic peptides, the calculations indicated that only ctuf-G and ctuf-D would exist in structural families with backbone conformations similar to those calculated for tuftsin. The structure motif common to these three peptides was determined to be a type IV β-turn centered at the Lys-Pro dipeptide. Furthermore, ctuf-D was predicted to exist in an additional conformational family with a type III β-turn centered about Lys-Pro. From these calculations, it was suggested that ctuf-G and ctuf-D would be biologically active and the introduction of cyclic conformational constraints should help to reduce the entropic penalty to peptide binding. Therefore, the advantages of these analogs

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†Abbreviations: PMN, polymorphonuclear leukocyte; ctuf, cyclo(Thr-Lys-Pro-Arg); ctuf-G, cyclo(Thr-Lys-Pro-Arg-Gly); ctuf-D, cyclo(Thr-Lys-Pro-Arg-Asp); ctuf-K, cyclo(Thr-Lys-Pro-Arg-Lys); NK, natural killer; AIDS, acquired immunodeficiency syndrome; DPPA, diphenylphosphoryl azide; Et_3N, triethylamine; ACN, acetonitrile; HFBA, heptafluorobutyric acid; BOP, benzotriazoyloxy-tris-(dimethylamino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; MeOH, methanol; FAB-MS (M + H), fast atom bombardment mass spectrometry (molecular mass + mass of hydrogen atom); and HBSS, Hanks' balanced salt solution.

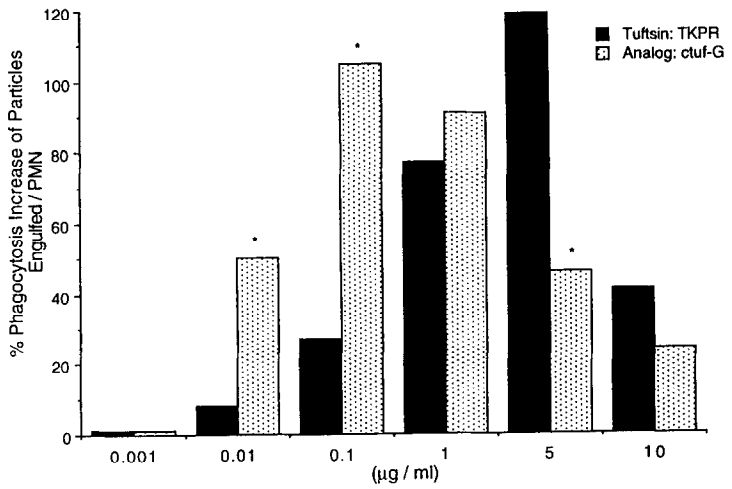

Fig. 1. Comparative human PMN phagocytosis stimulation activities of tuftsin and ctuf-G. PMN phagocytosis assay was performed as we described previously [21]. Each value is the mean of triplicate determinations. Key: (*) significantly different from tuftsin ($P < 0.05$).

are 2-fold: conformational considerations, and potential resistance against peptidases including attacks directed to the N- and C-terminal ends of tuftsin.

We synthesized these two cyclic analogs and tested them for phagocytosis stimulation ability. Indeed, ctuf-D was formed in two distinct diastereomers, separable by reverse-phase HPLC, named ctuf-Da and ctuf-Db based on the order of their elution from the HPLC column. We report here that compound ctuf-G and one of the isomers of ctuf-D were found to be more potent than linear tuftsin.

Materials and Methods

Chemical synthesis. Ctuf-G was synthesized according to the method of McMurray *et al.* [19] with the exception that cyclization was carried out with DPPA/Et_3N. The deprotected cyclic peptide was purified by reverse-phase HPLC using H_2O/ACN gradients containing 0.1% HFBA. FAB-MS (M + H) expected, 540.3; found, 540.4; amino acid analysis: Thr, 1.11; Lys, 1.00; Pro, 0.95; Arg, 1.01; Gly, 1.20. Ctuf-Da and ctuf-Db were prepared by classical solution phase techniques. Cyclization with BOP in the presence of $NaHCO_3$ resulted in the formation of two isomers. After removal of side-chain protection, the two isomers were separated by reverse-phase HPLC using H_2O/ACN gradients containing TFA. Ctuf-Da: FAB-MS (M + H) expected, 598.3; found, 598.3; amino acid analysis: Thr, 0.98; Lys, 1.00; Pro, 1.09; Arg, 1.15; Asp, 1.11. Ctuf-Db: FAB-MS (M + H) expected, 598.3; found, 598.3; amino acid analysis: Thr, 0.92; Lys 1.00; Pro, 1.09; Arg, 1.11; Asp, 1.10.

Phagocytosis assays. Compounds ctuf-G, ctuf-Da and ctuf-Db were converted to acetate salts from their HFBA and TFA salts by reverse-phase HPLC using 0.1 M ammonium phosphate/MeOH followed by another reverse-phase HPLC using 0.25 M ammonium acetate/MeOH mobile phases and lyophilized, as described by Amoscato *et al.* [20]. In the last HPLC, i.v. injectable water was used to minimize the endotoxin contamination. Endotoxin concentrations of final peptide solutions were measured with Limulus Ameobocyte Lysate Kit CL-1000 (Whittaker M. A. Bioproducts, Walkerville, MD) and were found to be <10 pg/mL. The amount of peptide in stock solutions was determined by amino acid analysis. Phagocytosis assays were carried out as described by Nishioka *et al.* [21]. In brief, heparinized human blood from a normal donor was mixed with dextran (Sigma Chemical Co., St. Louis, MO), and kept at 37° for 1 hr. The leukocyte-rich plasma was collected and centrifuged. The resulting pellet was washed with HBSS (Whittaker M. A. Bioproducts). The pellet was suspended in HBSS, layered over lymphocyte separation medium (Organon Teknika Corp., Durham, NC), and centrifuged. The sedimented cells were washed with HBSS. The contaminating erythrocytes were lysed by hypotonic treatment. The PMNs were washed with HBSS and adjusted to 1×10^6 viable cells/mL (viability >95% by trypan blue dye exclusion). PMNs (0.5×10^6/well) were plated in a 24-well plate (Costar Corp., Cambridge, MA), and placed in a 37° CO_2 incubator for 30 min to form the PMN monolayer. The supernatants were aspirated. Then peptide in 250 μL HBSS and 2.5×10^7 fluorescein-labeled polystylene latex microspheres with carboxylate groups (Fluoresbrite; 2.0 μm, Polyscience Inc., Warrington, PA) in 250 μL HBSS were added simultaneously to each well to obtain the desired final concentration of peptide and particle to PMN ratio of 50:1. After incubation at 37° for 15 min, the supernatants were aspirated and each well was washed with HBSS. To detach PMNs and remove the PMN surface-bound particles, each well was treated with 1 mL trypsin (0.25%, Hazleton Biologics, Inc., Lenexa, KS) at 37° for 15 min. The cells were then layered over fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) and centrifuged. The supernatants containing free particles were removed, and the cell pellets were suspended and fixed in 0.5 mL of 2% paraformaldehyde, and placed back into a well. The PMNs were examined by microscopy, and particles engulfed by PMNs were enumerated by counting 100 cells per well. The mean values from triplicates were used. With this method we have examined the correlation between microscopic countings and flow cytometric quantitation of phagocytosis and have demonstrated a correlation coefficient of 1.00. Student's *t*-test was used to determine significant differences between groups.

Results and Discussion

Peptides ctuf-G, ctuf-Da, and ctuf-Db were tested for their abilities to stimulate human PMN phagocytosis and were compared with tuftsin (Figs. 1 and 2). As with tuftsin, the cyclic analogs showed bell-shaped activity profiles. The optimum concentration of ctuf-G was 0.1 μg/mL, 50-fold

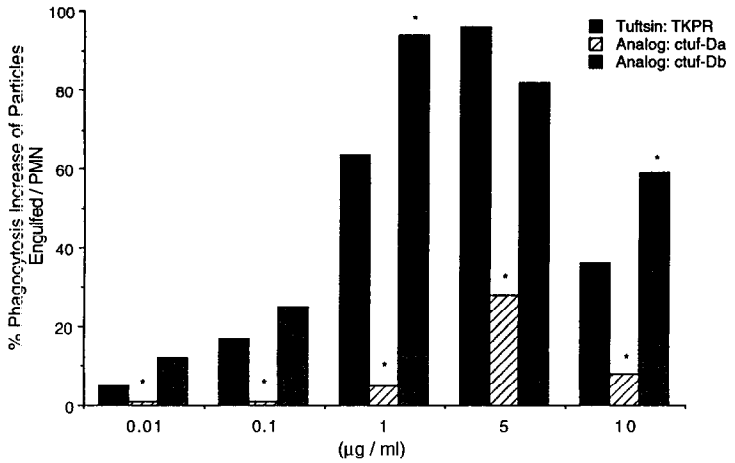

Fig. 2. Comparative human PMN phagocytosis stimulation activities of tuftsin with ctuf-Da and ctuf-Db. PMN phagocytosis assay was carried out as we described previously [21]. Each value is the mean of triplicate determinations. Key: (*) significantly different from tuftsin ($P < 0.05$).

less than that of tuftsin (5 µg/mL), and the degree of stimulation was similar (no significant difference). Ctuf-G was significantly more effective at lower concentrations than tuftsin. It is unknown at this time if this effect is an apparent increase in affinity for the tuftsin receptor due to the fact that this analog is held in a favorable conformation, as predicted by the modeling studies. It is also quite possible that the cyclic peptide is not degraded by the leucine aminopeptidase, thus making its effective concentration better reflect its prepared concentration.

It must be pointed out that the linear version of ctuf-G, Thr-Lys-Pro-Arg-Gly, had little effect on phagocytosis of PMNs [17]. Chipens *et al.* [17] prepared a cyclic version of Thr-Lys-Pro-Arg-Gly in which the side-chain of the Lys was coupled to the carboxy-terminus. At the concentration tested, this compound caused 30% of the stimulation of phagocytosis of tuftsin. In fact, all of the cyclic analogs prepared by these investigators, including ctuf, showed reduced phagocytosis activity compared with tuftsin.

Analog ctuf-Da was found to be virtually inactive with only slight activity at 5 µg/mL, showing a great deal of contrast with ctuf-Db. The optimum concentration of ctuf-Db was 1 µg/mL, 5-fold less than that of tuftsin, whereas the degree of stimulation was nearly the same. This result is in accord with the modeling studies that predicted two conformational families for this analog; one is similar to tuftsin and the other differs. It remains to be seen whether the conformations of our cyclic analogs agree with those predicted by O'Conner *et al.* [18].

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