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Small cyclic agonists of iron regulatory hormone hepcidin

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ABSTRACT

Minihepcidins are in vitro and in vivo active mimetics of iron-regulatory hormone hepcidin. They contain various unusual amino acids including: N-substituted, β-homo-, and D-amino acids with their combination depending on particular minihepcidin. In the current study, we sought to limit the use of unusual/-more expensive amino acids derivatives by peptide cyclization. Novel cyclic mimetics of hepcidin were synthesized and tested in vitro and showed activity at low nanomolar concentration. Nonetheless, the most active cyclic compound (mHS17) is approximately ten times less active than the parental minihepcidin PR73. Collectively, our findings suggest that cyclization is viable approach in the synthesis of hepcidin mimetics.

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Hepcidin (Fig. 1A), a 25 amino acids long peptide hormone, is a key regulator of iron homeostasis in verterbrates.[1] Its function is mediated through the membrane receptor/iron exporter, ferroportin (Fpn).[2] Hepcidin binds to Fpn which in turn causes the internalization of Fpn and its subsequent proteasomal degradation. In consequence, diminished levels of surface Fpn on duodenal enterocytes and hepatic and splenic macrophages restrict systemic iron availability, by decreasing both the absorption of dietary iron in duodenum and the release of recycled iron from macrophages.[3]

Hepcidin, ferroportin and related pathways are currently under scrutiny as novel target(s) in the search for new therapeutics for iron disorders, with several leading compounds in advanced stage of development.[4–6] Hepcidin itself has unfavorable pharmacological properties ($t_{1/2}$ <2.5 min)[7] and, because of its 4 disulfide bonds, it is notoriously difficult to synthesize.[8] We developed minihepcidins, rationally designed peptide-based hepcidin mimetics, with potent in vitro and in vivo bioactivity.[9,10] Minihepcidins are currently under commercial development by Merganser Biotech LLC. Data available to date indicate that minihepcidins could be useful in stand-alone and combination-therapy-regimen(s) for β-thalassemia and polycythemia vera as well as in the treatment of iron-dependent bacterial infections.[11–14]

Most active minihepcidins contain both natural and unusual amino acids, including N-substituted- and β-homo-amino acids, with the unusual amino acids conferring resistance to proteolysis.[15–17] The C-terminus of minihepcidins contains a conjugated lipid moiety (C_{16}) that increases plasma half-life and stabilizes the analog(s).[18–21] Lipidation may also anchor the peptide in the hydrophobic environment of lipid rafts, increasing its local concentration, and bioactivity.[21–25] Notably, lipidated *retro-inverso* minihepcidins were also synthesized and showed potent bioactivity.[9]

Due to their content of unusual amino acids, certain minihepcidins may be expensive to produce, especially in the large quantities. Additional costs result mainly from the use of β-homo-amino acids (β-homo-L-proline (βhPro) and β-homo-L-phenylalanine (βhPhe)) and the unusual amino acid, 3,3′-diphenyl-L-alanine (Dpa). Therefore we explored a different strategy that would avoid the use of the most expensive amino acid derivatives, produce active hepcidin mimetics, and decrease the cost of synthesis. We chose the peptide cyclization approach which has a long standing history.[26] Interestingly, head-to-tail cyclization of full length hepcidin with and without spacer-residue(s) was recently reported.[27] However these modifications resulted in inactive derivatives. In our case though, the starting point for modifications was the PR73 minihepcidin (Fig. 1B) rather than the full length hormone. Specifically, we employed the previously described S-alkylation method for the bridging of peptide(s) containing two strategically placed Cys residues with either symmetrical bis-halogeno-derivatives[28,29] or divinyl sulfone (DVS).[30] The use of this particular

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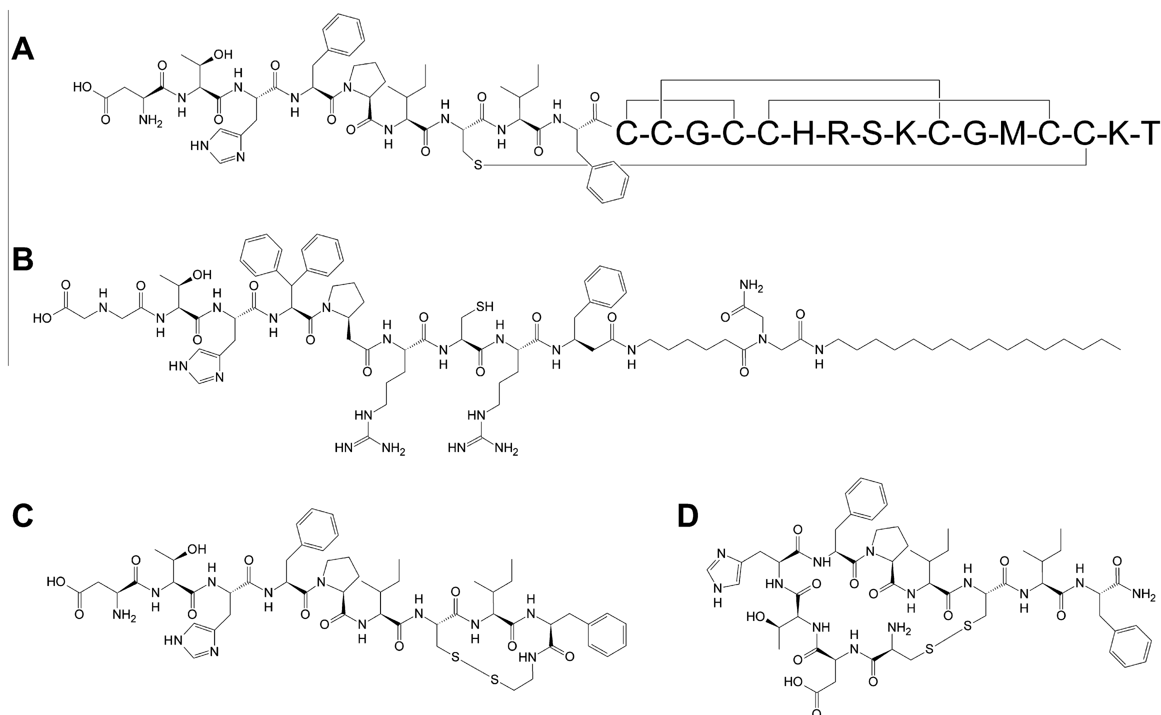

Figure 1. Comparison of structures of (A) human hepcidin, (B) minihepcidin PR73, (C) inactive disulfide-bond cyclized analog cycloDTHFPI(CIF-CONHCH$_2$CH$_2$S–), and (D) bioactive disulfide-bond cyclized analog cyclo(CDTHFPIC)IF-CONH$_2$.

cyclization method was prompted both by low cost and wide range of various alkylating analogs available, with an additional benefit of increased rigidity of the bridge that may stabilize the active conformer(s). The availability of various cysteine homologs: (L)Cys, (D)Cys, (L)homoCys, (D)homoCys, (L)Pen, and (D)Pen provides the option of 'fine tuning' of selected active derivatives. Moreover, all reactions can be carried out in solution without any protecting groups. Notably, this approach was already applied in peptide drug development[31], including phage display[32–42] as well as peptide–albumin[43,44] and peptide–antibody drug conjugates (ADCs).[45]

All linear peptides necessary for this study were synthesized by the solid phase method using CEM Liberty automatic microwave peptide synthesizer (CEM Corporation Inc., Matthews, NC), employing 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and commercially available amino acid derivatives and reagents (Chem-Impex International, Inc., Wood Dale, IL).[46,47] Since minihepcidins contain the biologically important cysteine residue in position 7[9,10] we used *S-tert*-butyl protected Cys-derivative (Cys(*t*Bu)) to avoid unwanted interference with S-alkylation of cysteines in these positions.

S-Alkylation/cyclization of reverse-phase high-performance liquid chromatography (RP-HPLC) purified linear analogs was performed using 2 different protocols, depending on the particular compound's structure. Generally, for S-alkylation of synthetic linear peptides which are moderately or well soluble in water, we employed the protocol described by Timmerman and coworkers.[28] These reactions were carried out at ambient temperature in 50 mM ammonium bicarbonate (NH_4HCO_3) dissolved in a mixture of acetonitrile (ACN) and water.[46] In case of hydrophobic peptides (e.g. lipidated analogs) we utilized previously described 1,1,3,3-tetramethylguanidine (TMG) driven reaction of thiol(s) containing compounds with bis-halogeno-derivatives in organic solvent/low molecular weight alcohol (preferably methanol),[48] that we adapted to peptides.[46] Since peptides are rarely well soluble in methanol, we used dimethyl sulfoxide (DMSO) as a solubilizing additive (up to 25%) and excess of TMG (final concn = 0.35%, vol/vol). In addition, we also synthesized bi- and tri-cyclic concatenated derivatives (mHS11–mHS13), in an attempt to benefit from the potential multivalency of binding to multimerized Fpn.

Cyclic crude analogs containing Cys(*t*Bu) residue(s) (position 7) were selectively deprotected using either modified TFMSA protocol or DMSO/TFA oxidation/dimerization followed by reduction with excess of tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 100 equiv/30 min).[46,49] Notably, an alternative *S-tert*-butyl deprotection protocol, utilizing 1 M HBF_4/thioanisole was also described.[50]

All synthesized cyclic analogs were purified by preparative RP-HPLC and characterized by matrix-assisted laser desorption ionization spectrometry (MALDI-MS) as well as analytical RP-HPLC[51] (see Table 1). Structural details for the synthesized analogs are presented in Figures 2, 4 and 5. Notably, for hydrophobic/lipidated analogs, an alternative synthetic protocol was also described.[52] Generally, cyclization leading to monocyclic mHS derivatives proceeded efficiently with 1,3-bis(bromomethyl)benzene and 2,6-bis(bromomethyl)pyridine giving superior results. On the other hand, analogs mHS8–10 were particularly difficult to obtain, regardless on used protocol giving estimated yields <5% (based on RP-HPLC purified material).

Bicyclic mHS11 was synthesized from its linear analog: Ac-CFPRXRFCFPRXRFC-CONH$_2$ (X = L-Cys(*St*Bu)) using 1,3,5-tris (bromomethyl)benzene as a bridging moiety.[28] Tricyclic counterparts were synthesized from Ac-CFPRXRFCFPRXRFCFPRXRFC-CONH$_2$ and either commercially available 1,2,4,5-tetrakis(bromomethyl) benzene[28] or pentaerythritol tetrakis(2-bromoacetate).[46,53] In this case, depending on bridging moiety structure/symmetry, single or multiple products (regioisomers) may be obtained. Single product of S-alkylation is possible if linker used in the process of S-alkylation possesses tetrahedral symmetry (T_d) (e.g., pentaerythritol scaffold). As expected, cyclization reaction with 1,2,4, 5-tetrakis(bromomethyl)benzene proceeded with low efficiency, which is most likely due to the generally rigid structure of the final

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