Supplementary Information

Pursuing orally bioavailable hepcidin analogues via cyclic N-methylated mini-hepcidins

Daniela Goncalves Monteiro[1], Johannes W. A. van Dijk[1], Randy Aliyanto[1], Eileen Fung[2], Elizabeta Nemeth[2], Tomas Ganz[2], K. Johan Rosengren[1], Richard J. Clark[1*]

[1]*School of Biomedical Sciences, Faculty of Medicine and Biomedical Sciences, University of Queensland, Chancellors Pl, Brisbane, QLD 4072, Australia*

[2]*David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, California, USA.*

*Corresponding author. Richard J. Clark

Tel.: +61 7 3365 1527

E-mail address: richard.clark@uq.edu.au

Table S1. Library of mini-hepcidin analogues that could not be tested.

#	Peptide	Sequence[1]	Calc. $[M+H]^+$	Obs. $[M+H]^+$	Yield[2]
1	Hepcidin	D T H F P I C I F C C G C C H R S K C G M C C K T	2789.42	$[M+3H^+]$ 930.5	5%
2	Hep9	D T H F P I C I F	1092.27	1092.5	20%
3	Hep9[Ser^1]	S T H F P I C I F	1064.26	1064.6	5-10%
4	Hep9[$MeIle^6$, $MeIle^8$]	D T H F P [*N*-Me I] C [*N*-Me I] F	1120.32	1120.4	5%
5	Hep9[Ser^1, $MeThr^2$, $MeIle^6$]	S [*N*-Me T] H F P [*N*-Me I] C I F	1092.31	1092.6	< 5%
6	Hep9[Ser^1, $MeThr^2$, $MeIle^8$]	S [*N*-Me T] H F P I C [*N*-Me I] F	1092.31	1092.8	< 5%
7	Hep9[Ser^1, $MeThr^2$, $MeIle^6$, $MeIle^8$]	S [*N*-Me T] H F P [*N*-Me I] C [*N*-Me I] F	1106.34	1106.5	< 3%
8	cHep9	c(D T H F P I C I F)	1074.26	1074.6	10%
9	cHep9[Ser^1]	c(S T H F P I C I F)	1046.24	1046.7	5%
10	cHep9-Gly_4	c(D T H F P I C I F G G G G)	1302.46	1302.8	5%
11	cHep9[$MePhe^4$, $MePhe^9$]	c(D T H [*N*-Me F] P I C I [*N*-Me F])	1102.31	1102.6	5%
12	cHep9[$MeIle^6$, $MeIle^8$]	c(D T H F P [*N*-Me I] C [*N*-Me I] F)	1102.31	1102.6	< 5%
13	cHep9[Ser^1, $MeThr^2$, $MeIle^8$]	c(S [*N*-Me T] H F P I C [*N*-Me I] F)	1074.30	1074.6	< 3%
14	Hep9[$MeThr^2$, $MeCys^7$]	D [*N*-Me T] H F P I [*N*-Me C] I F	1120.32	1120.8	Could not purify
15	Hep9[$MeThr^2$, $MeIle^6$, $MeCys^7$]	D [*N*-Me T] H F P [*N*-Me I] [*N*-Me C] I F	1134.35	-	Could not synthesise
16	Hep9[$MeThr^2$, $MeCys^7$, $MeIle^8$]	D [*N*-Me T] H F P I [*N*-Me C] [*N*-Me I] F	1134.35	-	Could not purify
17	Hep9[Ser^1, $MeThr^2$, $MeCys^7$]	S [*N*-Me T] H F P I [*N*-Me C] I F	1092.31	-	Could not synthesise
18	cHep9[$MeThr^2$, $MeCys^7$]	D [*N*-Me T] H F P I [*N*-Me C] I F	1102.31	1102.7	Could not purify
19	cHep9[$MeThr^2$, $MeIle^6$, $MeCys^7$]	D [*N*-Me T] H F P [*N*-Me I] [*N*-Me C] I F	1116.34	-	Could not synthesise

20	cHep9[MeThr[2], MeCys[7], MeIle[8]]	D [*N*-Me T] H F P I [*N*-Me C] [*N*-Me I] F	1116.34	1116.5	Could not purify
21	cHep9[Ser[1], MeThr[2], MeIle[6]]	S [*N*-Me T] H F P [*N*-Me I] C I F	1074.30	1074.6	Could not purify
22	cHep9[Ser[1], MeThr[2], MeIle[6], MeIle[8]]	S [*N*-Me T] H F P [*N*-Me I] C [*N*-Me I] F	1088.33	1088.6	Could not purify
23	cHep9[Ser[1], MeThr[2], MeCys[7]]	S [*N*-Me T] H F P I [*N*-Me C] I F	1074.30	-	Could not synthesise

[1]"c" corresponds to cyclic peptides and "*N*-Me" corresponds to *N*-methylated. [2]%Yield was calculated relative to crude product following HPLC purification.

Table S2. Liquid chromatography mass spectrometry (LC-MS) specifications for multiple reaction monitoring (MRM) protocols.

#	Compound	Obs. [M+xH$^+$]	MRM transitions	Retention time (min)
-	Atenolol	267.1	225.1 190.1 145.2	1.19
-	Methoxyverapamil	485.4	333.4 165.2 150.1	6.79
2	Hep 9	1092.7	501.1 354.2 336.0	6.54
4	Hep9[MeIle^6, MeIle^8]	1120.7	372.4 245.2 217.5	6.48
6	Hep9[Ser^1, MeThr^2, MeIle^8]	1092.0	314.4 285.5 203.3	6.48
8	cHep9	1074.7	314.2 216.9 183.2	6.96
12	cHep9[MeIle^6, MeIle^8]	1102.9	294.4 217.1 156.2	7.15
13	cHep9[Ser^1, MeThr^2, MeIle^8]	1074.5	314.3 216.8 209.1	6.98

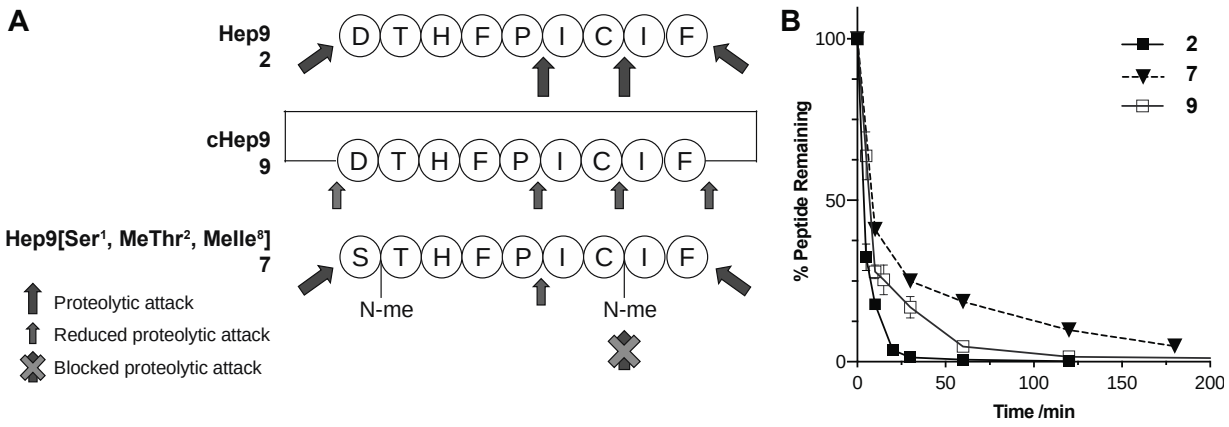

Figure S1. Effect of macrocyclisation and *N*-methylation on the stability of selected mini-hepcidin derivatives. **A**) Illustration of sites most susceptible to enzymatic attack in mini-hepcidin derivatives. **B**) Serum stability analysis of selected mini-hepcidins. Human male serum was centrifuged at 20,000 g for 10 minutes and the clear part of the supernatant was then diluted to 25% (v/v) with Milli-Q® water and incubated at 37ºC for 15 minutes. Each peptide sample was dissolved in triplicate at a final concentration of 20 μM in pre-incubated 25% serum, or Milli-Q® water as a control. Aliquots of 100 μL were removed at different time points and quenched with 100 μL of 15% trichloroacetic acid (TCA), followed by incubation on ice for 30 min and centrifugation at 14,000 g for 5 minutes. Supernatant was then removed and transferred into vials for liquid chromatography mass spectrometry (LC-MS) analysis (50 μL injection and multiple reaction monitoring (MRM) protocol). Error bars indicate SEM, n=3. Comparison between Hep9, ***2***, Hep9[Ser^{1}, MeThr^{2}, MeIle^{8}], ***7***, and cHep9, ***9***.