(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 July 2016 (07.07.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/109363 A1

(51) International Patent Classification:
C07K 7/06 (2006.01)

(21) International Application Number:
PCT/US2015/067545

(22) International Filing Date:
27 December 2015 (27.12.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/097,429 29 December 2014 (29.12.2014) US

(71) Applicant: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, Twelfth Floor, Oakland, California 94607-5200 (US).

(72) Inventors: **RUCHALA, Piotr**; c/o UCLA Office of Intellectual Property Administration, 11000 Kinross Ave., Ste 200 MC 140607, Los Angeles, California 90095-1406 (US). **GANZ, Tomas**; c/o UCLA Office of Intellectual Property Administration, 11000 Kinross Ave., Ste 200 MC 140607, Los Angeles, California 90095-1406 (US). **NEMETH, Elizabeta**; c/o UCLA Office of Intellectual Property Administration, 11000 Kinross Ave., Ste 200 MC 140607, Los Angeles, California 90095-1406 (US).

(74) Agent: **SUNDBY, Suzannah K.**; Canady + Lortz LLP, 1050 30th Street, NW, Washington, District of Columbia 20007 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: S-Alkylated Hepcidin Peptides and Methods of Making and Using Thereof

(57) Abstract: Disclosed herein S-alkylated hepcidin peptides and methods of making and using thereof. In some embodiments, the present invention is directed to an S-alkylated hepcidin peptide having the following Structural Formula IA or IB. In some embodiments, the present invention is directed to a composition comprising at least one S-alkylated hepcidin peptide of the present invention. In some embodiments, the present invention is directed to a method of binding a ferroportin or inducing ferroportin internalization and degradation which comprises contacting the ferroportin with at least one S-alkylated hepcidin peptide of the present invention. In some embodiments, the present invention is directed to a kit comprising at least one S-alkylated hepcidin peptide.

WO 2016/109363 A1

S-ALKYLATED HEPCIDIN PEPTIDES AND METHODS OF MAKING AND USING THEREOF

[0001] CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims the benefit of U.S. Application No. 62/097,429, filed December 29, 2014, which is herein incorporated by reference in its entirety.

[0003] ACKNOWLEDGEMENT OF GOVERNMENT SUPPORT

[0004] This invention was made with Government support under DK090554, awarded by the National Institutes of Health. The Government has certain rights in the invention.

[0005] REFERENCE TO A SEQUENCE LISTING SUBMITTED VIA EFS-WEB

[0006] The content of the ASCII text file of the sequence listing named "20151227_034044_155WO1_seq_ST25" which is 41.7 kb in size was created on December 27, 2015 and electronically submitted via EFS-Web herewith the application is incorporated herein by reference in its entirety.

[0007] BACKGROUND OF THE INVENTION

[0008] 1. FIELD OF THE INVENTION

[0009] The present invention generally relates to S-alkylated hepcidin peptides and methods of making and using thereof.

[0010] 2. DESCRIPTION OF THE RELATED ART

[0011] Hepcidin, a peptide hormone produced by the liver, is a regulator of iron homeostasis in humans and other mammals. Hepcidin acts by binding to its receptor, the iron export channel ferroportin, and causing its internalization and degradation. Human hepcidin is a 25–amino acid peptide (Hep25). See Krause et al. (2000) FEBS Lett 480:147-150, and Park et al. (2001) J Biol Chem 276:7806-7810. The structure of the bioactive 25–amino acid form of hepcidin is a simple hairpin with 8 cysteines that form 4 disulfide bonds as described by Jordan et al. (2009) J Biol Chem 284:24155-67. The N terminal region is required for iron-regulatory function, and deletion of 5 N-terminal amino acid residues results in a loss of iron-regulatory function. See Nemeth et al. (2006) Blood 107:328-33.

[0012] Abnormal hepcidin activity is associated with iron overload diseases which include hereditary hemochromatosis and iron-loading anemias and myelodysplasia. Hereditary hemochromatosis (HH) is a genetic iron overload disease that is mainly caused by hepcidin deficiency, or very rarely by hepcidin resistance. This allows excessive absorption of iron from the diet and development of iron overload. Clinical manifestations

of HH may include liver disease (hepatic cirrhosis, hepatocellular carcinoma), diabetes, and heart failure. Currently, the only treatment for HH is regular phlebotomy, which is effective but very burdensome for the patients.

[0013] Iron-loading anemias are hereditary anemias with ineffective erythropoiesis such as β-thalassemia, which are accompanied by severe iron overload. Complications from iron overload are the main cause of morbidity and mortality for these patients. Hepcidin deficiency is the main cause of iron overload in untransfused patients, and contributes to iron overload in transfused patients. The current treatment for iron overload in these patients is iron chelation which is very burdensome, sometimes ineffective and accompanied by frequent side effects.

[0014] Mini-hepcidin peptides disclosed in WO 2010/065815 and modified mini-hepcidin peptides disclosed in WO 2013/086143 exhibit hepcidin activity and can be used to modulate iron metabolism and treat diseases of iron metabolism. Many of these mini-hepcidin peptides contain an unprotected free-cysteine residue, e.g., at the A7 amino acid position. Unfortunately, peptide-based therapeutics that contain and/or release free sulfhydryl group(s) can be problematic as they may exhibit (1) decreased stability associated with inherent free-thiol reactivity (S-alkylation/oxidation), and/or (2) dermatological side effects (e.g. skin eruptions).

[0015] SUMMARY OF THE INVENTION

[0016] In some embodiments, the present invention is directed to an S-alkylated hepcidin peptide having the following Structural Formula IA or IB

A1-A2-A3-A4-A5-A6-A7-A8-A9-A10 IA

A10-A9-A8-A7-A6-A5-A4-A3-A2-A1 IB

wherein

A1 is Asp, D-Asp, Glu, D-Glu, pyroglutamate, D-pyroglutamate, Gln, D-Gln, Asn, D-Asn, or an unnatural amino acid commonly used as a substitute thereof such as bhAsp, Ida, Ida(NHPal), and N-MeAsp, preferably Ida and N-MeAsp;

A2 is Thr, D-Thr, Ser, D-Ser, Val, D-Val, Ile, D-Ile, Ala, D-Ala or an unnatural amino acid commonly used as a substitute thereof such as Tle, Inp, Chg, bhThr, and N-MeThr;

A3 is His, D-His, Asn, D-Asn, Arg, D-Arg, or an unnatural amino acid commonly used as a substitute thereof such as L-His(π-Me), D-His(π-Me), L-His(τ-Me), or D-His(τ-Me);

A4 is Phe, D-Phe, Leu, D-Leu, Ile, D-Ile, Trp, D-Trp, Tyr, D-Tyr, or an unnatural amino acid commonly used as a substitute thereof such as Phg, bhPhe, Dpa, Bip, 1Nal, 2Nal, bhDpa, Amc, PheF5, hPhe, Igl, or cyclohexylalanine, preferably Dpa;

A5 is Pro, D-Pro, Ser, D-Ser, or an unnatural amino acid commonly used as a substitute thereof such as Oic, bhPro, trans-4-PhPro, cis-4-PhPro, cis-5-PhPro, and Idc, preferably bhPro;

A6 is Arg, D-Arg, Ile, D-Ile, Leu, D-Leu, Thr, D-Thr, Lys, D-Lys, Val, D-Val, or an unnatural amino acid commonly used as a substitute thereof such as D-Nω,ω-dimethyl-arginine, L-Nω,ω-dimethyl-arginine, D-homoarginine, L-homoarginine, D-norarginine, L-norarginine, citrulline, a modified Arg wherein the guanidinium group is modified or substituted, Norleucine, norvaline, bhIle, Ach, N-MeArg, and N-MeIle, preferably Arg;

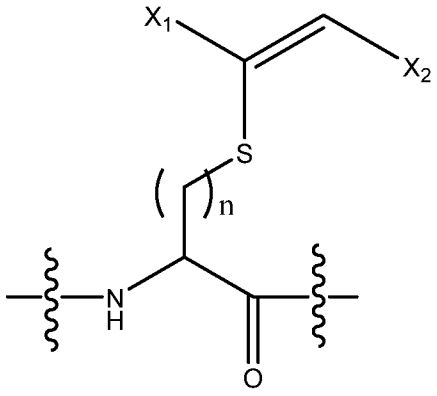
A7 is Cys, D-Cys, Ser, D-Ser, Ala, D-Ala, or an unnatural amino acid commonly used as a substitute thereof such as Cys(S-tBut), homoCys, Pen, (D)Pen, preferably S-tertiary butyl-cysteine, Cys(S-S-Pal), Cys(S-S-cysteamine-Pal), Cys(S-S-Cys-NHPal), and Cys(S-S-Cys);

A8 is Arg, D-Arg, Ile, D-Ile, Leu, D-Leu, Thr, D-Thr, Lys, D-Lys, Val, D-Val, or an unnatural amino acid commonly used as a substitute thereof such as D-Nω,ω-dimethyl-arginine, L-Nω,ω-dimethyl-arginine, D-homoarginine, L-homoarginine, D-norarginine, L-norarginine, citrulline, a modified Arg wherein the guanidinium group is modified or substituted, Norleucine, norvaline, bhIle, Ach, N-MeArg, and N-MeIle, preferably Arg;

A9 is Phe, D-Phe, Leu, D-Leu, Ile, D-Ile, Tyr, D-Tyr, Trp, D-Trp, Phe-R^a, D-Phe-R^a, Dpa-R^a, D-Dpa-R^a, Trp-R^a, bhPhe-R^a, or an unnatural amino acid commonly used as a substitute thereof such as PheF5, N-MePhe, benzylamide, 2-aminoindane, bhPhe, Dpa, Bip, 1Nal, 2Nal, bhDpa, and cyclohexylalanine, which may or may not have R^a linked thereto, preferably bhPhe and bhPhe-R^a, wherein R^a is palmitoyl-PEG-, wherein PEG is PEG11 or miniPEG3, palmitoyl-PEG-PEG, wherein PEG is PEG11 or miniPEG3, butanoyl (C4)-PEG11-, octanoyl (C8, Caprylic)-PEG11-, palmitoyl (C16)-PEG11-, or tetracosanoyl (C24, Lignoceric)-PEG11-; and

A10 is Cys, D-Cys, Ser, D-Ser, Ala, D-Ala, or an unnatural amino acid such as Ida, Ida(NHPal)Ahx, and Ida(NBzl2)Ahx; and

at least one of the amino acid residues A1 to A10 has the following Structural Formula A:



(A)

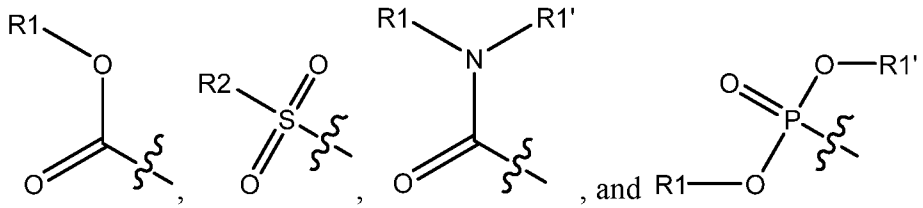
wherein

n is 1 or 2 and one or more of the hydrogens bonded to the Cn atom(s) may be substituted with a (C_1-C_3)alkyl,

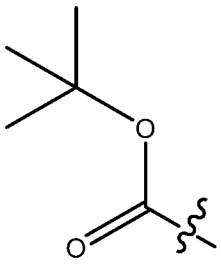
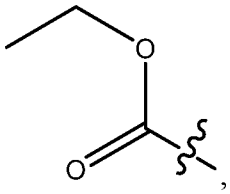
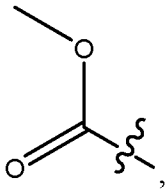
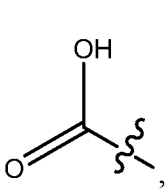
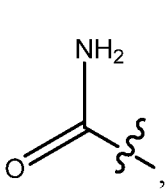
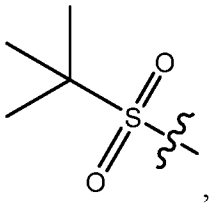
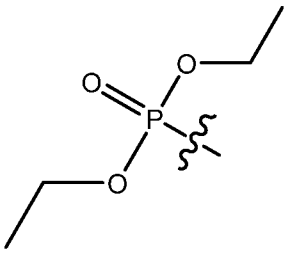
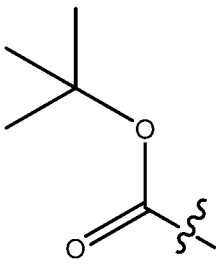
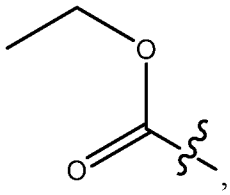
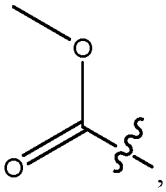
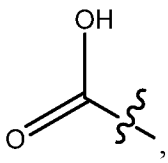
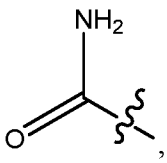
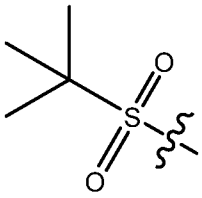
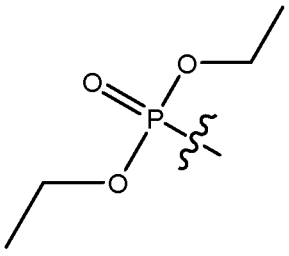
X_1 and X_2 are each independently selected from the group consisting of H, alkyl, alkoxy, alkoxycarbonyl, cycloalkyl, aryl, heteroaryl, heterocycloalkyl, acyl, sulfonyl, alkylsulfonyl, alkylamino, alkylaminocarbonyl, dialkylaninocarbonyl, carboxyl, and carbamoyl;

wherein the carboxy-terminal amino acid is in amide or carboxy- form; and

wherein A1, A1 to A2, A10, or a combination thereof are optionally absent. In some embodiments, the S-alkylated hepcidin peptide has an amino acid sequence selected from SEQ ID NOs: 1-101 with at least one amino acid substitution, said at least one amino acid substitution has the Structural Formula A. In some embodiments, the amino acid residue having Structural Formula A corresponds to a thiol containing amino acid of SEQ ID NOs: 1-101. In some embodiments, the amino acid residue having Structural Formula A is A7. In some embodiments, A1 is Ida, A2 is Thr, A3 is His, A4 is Dpa, A5 is bhPro, A6 is Arg, A8 is Arg, A9 is bhPhe, and A10 is Ahx-Ida(NHPal). In some embodiments, X_1 and X_2, are each independently selected from the group consisting of H, phenyl,



, wherein R1 and R1' are each independently selected from the group consisting of H, methyl, (C_2)alkyl, (C_3)alkyl, (C_4)alkyl, (C_1-C_5)alkyl, (C_6)alkyl, (C_7)alkyl, (C_8)alkyl, (C_9)alkyl, and (C_{10})alkyl; and R2 is –NR1R1', methyl, (C_2)alkyl, (C_3)alkyl, (C_4)alkyl, (C_1-C_5)alkyl, (C_6)alkyl, (C_7)alkyl, (C_8)alkyl, (C_9)alkyl, and (C_{10})alkyl. In some embodiments, R1 and R1' are each independently selected from the group consisting of H, methyl, ethyl, isopropyl, and tert-butyl. In some embodiments, X_1 and X_2 are each independently

selected from the group consisting of H, phenyl,  ,  ,  ,  ,  ,  , and  . In some embodiments, X_1 and X_2 are (a) both  , (b) both  , (c) both  , (c) H and  , respectively, (d) phenyl and  , respectively, (e) both  , or (f) both  .

[0017] In some embodiments, the present invention is directed to a composition comprising at least one S-alkylated hepcidin peptide of the present invention, e.g., an S-alkylated hepcidin peptide as set forth in paragraph [0016] above.

[0018] In some embodiments, the present invention is directed to a method of binding a ferroportin or inducing ferroportin internalization and degradation which comprises contacting the ferroportin with at least one S-alkylated hepcidin peptide of the present invention, e.g., an S-alkylated hepcidin peptide as set forth in paragraph [0016] above, or a composition thereof.

[0019] In some embodiments, the present invention is directed to a kit comprising at least one S-alkylated hepcidin peptide of the present invention, e.g., an S-alkylated hepcidin

peptide as set forth in paragraph [0016] above, or a composition thereof packaged together with a reagent, a device, instructional material, or a combination thereof.

[0020] In some embodiments, the present invention is directed to a complex comprising at least one S-alkylated hepcidin peptide of the present invention, e.g., an S-alkylated hepcidin peptide as set forth in paragraph [0016] above, bound to a ferroportin or an antibody.

[0021] In some embodiments, the present invention is directed to a method of treating a disease of iron metabolism in a subject which comprises administering at least one S-alkylated hepcidin peptide of the present invention, e.g., an S-alkylated hepcidin peptide as set forth in paragraph [0016] above, or a composition thereof to the subject. In some embodiments, the disease of iron metabolism is an iron overload disease. In some embodiments, the present invention is directed to the use of one or more S-alkylated hepcidin peptides of the present invention, e.g., an S-alkylated hepcidin peptide as set forth in paragraph [0016] above, or a composition thereof for the manufacture of a medicament for treating a disease of iron metabolism and/or lowering the amount of iron in a subject in need thereof. In some embodiments, the present invention is directed to one or more S-alkylated hepcidin peptides of the present invention, e.g., an S-alkylated hepcidin peptide as set forth in paragraph [0016] above, or a composition thereof for use in treating a disease of iron metabolism and/or lowering the amount of iron in a subject in need thereof. In some embodiments, the present invention is directed to the use of one or more S-alkylated hepcidin peptides of the present invention, e.g., an S-alkylated hepcidin peptide as set forth in paragraph [0016] above, or a composition thereof for the manufacture of a medicament for treating a disease of iron metabolism and/or lowering the amount of iron in a subject in need thereof, wherein the medicament is prepared to be administered at an effective daily dose as a single daily dose or as divided daily doses. In some embodiments, the effective daily dose is about 10-500 μg/kg/day and the medicament is formulated for subcutaneous injection. In some embodiments, the effective daily dose is about 10-1000 μg/kg/day and the medicament is formulated for oral, pulmonary, or mucosal administration. In some embodiments, the subject is a mammal. In some embodiments, the subject is human.

[0022] Both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed. The accompanying drawings are included to provide a further understanding of the invention and are incorporated in and constitute part of this

specification, illustrate several embodiments of the invention, and together with the description serve to explain the principles of the invention.

[0023] DESCRIPTION OF THE DRAWINGS

[0024] This invention is further understood by reference to the drawings wherein:

[0025] Figure 1 schematically shows the synthetic scheme for S-alkylation of hepcidin peptides using PR73 as an example.

[0026] Figure 2 shows the general structure of S-derivatized PR73 analogs. The structures in the top row are the structures which replace that encompassed in the circle shown in the bottom structure (PR73 (SEQ ID NO: 90)).

[0027] Figures 3A and 3B are graphs comparing the *in vitro* and *in vivo* activity of PR73 and PR73SH. Figure 3A are representative examples of *in vitro* dose response curves obtained for PR73 and PR73SH analogs using ferroportin degradation assay. Figure 3B are bar graphs comparing the *in vivo* activity of PR73 and PR73SH at 6, 24, and 48 hour time-points after administration by intraperitoneal injection.

[0028] DETAILED DESCRIPTION OF THE INVENTION

[0029] As used herein, "hepcidin peptides" refers to mini-hepcidin peptides disclosed in WO 2010/065815 and modified mini-hepcidin peptides disclosed in WO 2013/086143. As used herein, a "thiol-containing hepcidin peptide" refers to a hepcidin peptide having an amino acid residue containing a free thiol group (–SH). Thiol-containing hepcidin peptides include those having an unprotected free cysteine residue at amino acid position 7 as set forth in the structural formulas of WO 2010/065815 and WO 2013/086143. WO 2010/065815 and WO 2013/086143 are herein incorporated by reference in their entirety.

[0030] The present invention provides S-alkylated hepcidin peptides and methods of making and using thereof. As used herein, an "S-alkylated hepcidin peptide" refers to a peptide in which the hydrogen of the free thiol group (–SH) of a thiol-containing hepcidin peptide is substituted by S-alkylation.

[0031] As disclosed herein, 1,2-double substituted vinyl-sulfides, which may be efficiently synthesized from corresponding electron-deficient alkynes and unprotected free-cysteine containing peptides in aqueous media, were used as a protecting moiety. See Figure 1. Specifically, S-alkylated hepcidin peptides, PR73SA-PR73SH, were derived in a one-step reaction from parental peptide, PR73, as a representative thiol-containing hepcidin peptide. PR73 was synthesized as previously described. See Preza, et al. (2011) J. Clin. Invest., 121, 4880. Briefly, PR73 was assembled by the solid phase method using CEM Liberty automatic microwave peptide synthesizer (CEM Corporation Inc.,

Matthews, NC), applying 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and commercially available amino acid derivatives and reagents (EMD Biosciences, San Diego, CA and Chem-Impex International, Inc., Wood Dale, IL). Rinkamide- MBHA resin (EMD Biosciences, San Diego, CA) was used as a solid support. Peptide was cleaved from resin using modified reagent K (TFA 94% (v/v); phenol, 2% (w/v); water, 2% (v/v); TIS, 1% (v/v); EDT, 1% (v/v); 2 hours) and precipitated by addition of ice-cold diethyl ether. The peptide was purified by preparative reverse-phase high performance liquid chromatography (RP-HPLC) and its purity evaluated by matrix-assisted laser desorption ionization spectrometry (MALDI-MS) as well as analytical RP-HPLC.

[0032] PR73 was solubilized in 80% 1,4-dioxane in water, containing 50 mM N-methylmorpholine (NMM) (about 2 mg/mL) and subsequently a given electron-deficient alkyne was added (2 eq.). The S-alkylated hepcidin peptides as exemplified herein, and the given electron-deficient alkynes used to produce the exemplified S-alkylated hepcidin peptides are: (1) PR73SA - Di-tert-butyl acetylenedicarboxylate, (2) PR73SB - Diethyl acetylenedicarboxylate, (3) PR73SC - Dimethyl acetylenedicarboxylate, (4) PR73SD - Acetylenedicarboxylic acid, (5) PR73SE - 2-Phenylethynesulfonamide (Pifithrin-μ), (6) PR73SF - 1,2-Bis(tert-butylsulfonyl)acetylene, (7) PR73SG - Acetylenedicarboxamide, and (8) PR73SH - Bis(diethoxyphosphoryl)acetylene. Figure 2 shows the chemical structures of the exemplified S-alkylated hepcidin peptides. The mixture was vigorously stirred for 25 minutes at room temperature and subsequently lyophilized. A solid residue was obtained and purified by preparative reverse-phase high performance liquid chromatography (RP-HPLC) and its purity was evaluated by matrix-assisted laser desorption ionization spectrometry (MALDI-MS) as well as analytical RP-HPLC. See Table 1.

Table 1
Analytical and *in vitro* activity data for S-alkylated PR73 analogs

Peptide	Composition	MW Calc/Found	R_T [min]	EC_{50} [nM] TREX-hFpn-GFP cells
PR73	$C_{86}H_{133}N_{21}O_{15}S$	1733.19/1734.34	47.11	4.2±0.3
PR73SA	$C_{98}H_{151}N_{21}O_{19}S$	1959.46/1959.80	52.47	6.3±1.2
PR73SB	$C_{94}H_{143}N_{21}O_{19}S$	1903.35/1904.58	49.44	10.4±1.2
PR73SC	$C_{92}H_{139}N_{21}O_{19}S$	1875.30/1876.60	48.32	12.6±1.8
PR73SD	$C_{89}H_{135}N_{21}O_{17}S$	1803.24/1803.66	46.60	218.1±13.4
PR73SE	$C_{94}H_{140}N_{22}O_{17}S_2$	1914.40/1915.02	48.52	34.0±5.4
PR73SF	$C_{96}H_{151}N_{21}O_{19}S_3$	1999.56/1999.80	52.89*	10.0±3.4
PR73SG	$C_{90}H_{137}N_{23}O_{17}S$	1845.28/1846.59	49.33*	8.4±2.5
PR73SH	$C_{96}H_{153}N_{21}O_{21}P_2S$	2031.40/2031.33	52.18*	1.1±0.1

Analytical RP-HPLC was performed using an analytical reversed-phase C4 XBridge™ BEH300 column, 4.6 x 150 mm, 3.5 μm (Waters, Milford, MA), or (*) an analytical reversed-phase C18 SymmetryShield™ column, 4.6 x 250 mm, 5 μm (Waters, Milford, MA).

[0033] The S-alkylated hepcidin peptides were tested *in vitro* using a previously described cellular assay based on Fpn degradation. See e.g., Nemeth, et al. (2006) Blood 107: 328. Briefly, HEK293:TREX-Fpn-GFP, a cell line stably transfected with the human ferroportin-GFP construct under the control of doxycycline-inducible promoter, was plated on poly-D-lysine–coated plates in the presence of 20 μM FAC. Fpn expression was induced with 500 ng/mL doxycycline treatment for 24 hours. Then, doxycycline was washed off, and cells were treated with peptides for 24 hours. Cells were then trypsinized and resuspended at 1 x 10^6 cells/mL, and the intensity of green fluorescence was analyzed by flow cytometry using FACScan (fluorescence activated cell scanner) Analytic Flow Cytometer (Becton Dickinson, San Jose, CA) with CellQuest version 3.3 software. Cells not induced with doxycycline to express Fpn-GFP were used to establish a gate to exclude background fluorescence. Cells induced with doxycycline, but not treated with any peptides, were used as the positive control. Each peptide treatment was repeated independently 3 to 6 times. The results were expressed as a fraction of the activity of Hep25, according to Formula 1, (Fx - FHep25)/(Funtreated - FHep25), where F is the mean of the gated green fluorescence and x is the peptide. The results are summarized in Table 1. Generally, the S-alkylated hepcidin peptides showed high potency in the low nanomolar range. PR73SH, however, showed bioactivity (EC_{50} = 1.1 ± 0.1 nM) that is higher than the parental PR73 (EC_{50} = 4.2 ± 0.3 nM). Interestingly, the chemical synthesis of the S-alkylated hepcidin peptides does not appear to have a significant impact on bioactivity, rather the overall steric hindrance plays a significant role, with the most bulky substituents having hepcidin activity that is the same or better than Hep25. Hydrophobicity may also play a role, as activity increases in the carboxy-esters-substituent(s) order: $–CH_3$ < $–C_2H_5$ < $–C_4H_9$ (PR73SC < PR73SB < PR73SA).

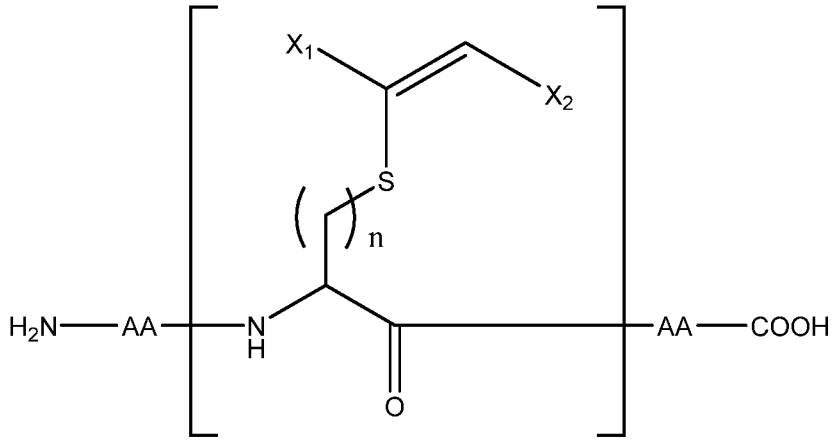
[0034] Additionally, the geometry of the vinyl substituents (planar versus tetrahedral) does not appear to significantly influence activity, as planar analog PR73SA has fairly similar potency to its tetragonal counterpart (PR73SF). Considering that remaining tetragonal analog PR73SH shows highest activity, and the fact that all 3 analogs (e.g., PR73SA, PR73SF and PR73SH) are chemically fairly similar having the same number of substituent(s)-carbon-atoms (2 x 4 = 8), overall volume/space occupied by S-attached moiety appears again as important factor, with the activity increasing from most compact

(PR73SF) to most bulky (PR73SH) substituent(s). Consistently, PR73SD, which has the most hydrophilic and least bulky substituent, shows the lowest potency (EC_{50} = 218.1 ± 13.4 nM).

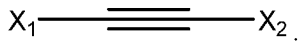
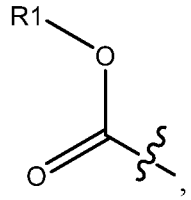
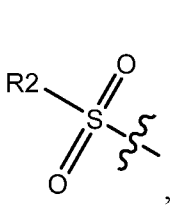
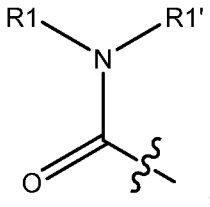
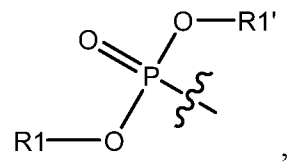
[0035] Based on *in vitro* results, PR73SH was selected as a suitable candidate for animal studies, which were carried out as previously described. See Preza, et al. (2011) J. Clin. Invest. 121:4880; Ramos, et al. (2012) Blood 120:3829; and Nemeth, et al. (2006) Blood 107:328. Animal studies were approved by the Animal Research Committee at UCLA. Briefly, C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained on NIH 31 rodent diet (iron content 336 mg/kg; Harlan Teklad, Indianapolis, IA). Mice were injected intraperitoneally either with 100 μL PBS (control) or with 50 or 100 nmoles peptide in 100 μL PBS. Mice were killed 6, 24, and 48 hours later, blood was collected by cardiac puncture, and serum was separated using Microtainer tubes (Becton Dickinson, Franklin Lakes, NJ). Serum iron was determined by using a colorimetric assay (Diagnostic Chemicals, Oxford, CT), which was modified for the microplate format so that 50 μL serum was used per measurement. See Nemeth, et al. (2004) J. Clin. Invest. 113(9): 1271-1276. The results were expressed as the percentage of decrease in serum iron when compared with the average value of serum iron levels in PBS-injected mice.

[0036] *In vivo* activity of PR73SH and PR73 was compared by assaying serum iron levels at 3 time points: (6, 24, and 48 hours) and concentrations that were previously shown to be sufficient for PR73 to exert potent bioactivity (50-100 nmoles/mouse). PR73SH activity was similar to the parental PR73 activity profile, with decreased serum iron observed at 6 and 24 hour time points, but not at the 48 hour time point (Figure 3B). Since no significant activity difference between PR73 and PR73SH was observed in either, *in vitro* or *in vivo* experiments, S-alkylated hepcidin peptides may be used to diseases of iron metabolism, such as iron overload disease, in subjects.

[0037] Therefore, in some embodiments, the S-alkylated hepcidin peptides according to the present invention comprise an S-alkylated cysteine residue having the bracketed structure set forth in Structural Formula I:



(I)

wherein n is 1 or 2 and one or more of the hydrogens bonded to the Cn atom(s) may be substituted with a (C_1-C_3)alkyl, AA represent the amino acid residues flanking the bracketed S-alkylated cysteine residue (in brackets) and X_1 and X_2, may be the same or different, and are the X_1 and X_2 groups of an electron-deficient alkyne having the formula  . In some embodiments X_1 and X_2 are each independently selected from the group consisting of H, alkyl, alkoxy, alkoxycarbonyl, cycloalkyl, aryl, heteroaryl, heterocycloalkyl, acyl, sulfonyl, alkylsulfonyl, alkylamino, alkylaminocarbonyl, dialkylaninocarbonyl, carboxyl, and carbamoyl. In some embodiments, X_1 and X_2, are each independently selected from the group consisting of H, phenyl,  ,  ,  , and  , wherein R1 and R1' are each independently selected from the group consisting of H, methyl, (C_2)alkyl, (C_3)alkyl, (C_4)alkyl, (C_1-C_5)alkyl, (C_6)alkyl, (C_7)alkyl, (C_8)alkyl, (C_9)alkyl, and (C_{10})alkyl; and R2 is –NR1R1', methyl, (C_2)alkyl, (C_3)alkyl, (C_4)alkyl, (C_1-C_5)alkyl, (C_6)alkyl, (C_7)alkyl, (C_8)alkyl, (C_9)alkyl, or (C_{10})alkyl. In some embodiments, R1 and R1' are each independently selected from the group consisting of H, methyl, ethyl, isopropyl, and tert-butyl. In some embodiments, the S-alkylated cysteine residue is at amino acid position 7 corresponding to the structural formulas of WO 2010/065815 and WO 2013/086143.

[0038] In some embodiments, the S-alkylated hepcidin peptides according to the present invention have the following Structural Formula IA or IB

A1-A2-A3-A4-A5-A6-A7-A8-A9-A10 IA

A10-A9-A8-A7-A6-A5-A4-A3-A2-A1 IB

wherein

A1 is Asp, D-Asp, Glu, D-Glu, pyroglutamate, D-pyroglutamate, Gln, D-Gln, Asn, D-Asn, or an unnatural amino acid commonly used as a substitute thereof such as bhAsp, Ida, Ida(NHPal), and N-MeAsp, preferably Ida and N-MeAsp;

A2 is Thr, D-Thr, Ser, D-Ser, Val, D-Val, Ile, D-Ile, Ala, D-Ala or an unnatural amino acid commonly used as a substitute thereof such as Tle, Inp, Chg, bhThr, and N-MeThr;

A3 is His, D-His, Asn, D-Asn, Arg, D-Arg, or an unnatural amino acid commonly used as a substitute thereof such as L-His(π-Me), D-His(π-Me), L-His(τ-Me), or D-His(τ-Me);

A4 is Phe, D-Phe, Leu, D-Leu, Ile, D-Ile, Trp, D-Trp, Tyr, D-Tyr, or an unnatural amino acid commonly used as a substitute thereof such as Phg, bhPhe, Dpa, Bip, 1Nal, 2Nal, bhDpa, Amc, PheF5, hPhe, Igl, or cyclohexylalanine, preferably Dpa;

A5 is Pro, D-Pro, Ser, D-Ser, or an unnatural amino acid commonly used as a substitute thereof such as Oic, bhPro, trans-4-PhPro, cis-4-PhPro, cis-5-PhPro, and Idc, preferably bhPro;

A6 is Arg, D-Arg, Ile, D-Ile, Leu, D-Leu, Thr, D-Thr, Lys, D-Lys, Val, D-Val, or an unnatural amino acid commonly used as a substitute thereof such as D-Nω,ω-dimethyl-arginine, L-Nω,ω-dimethyl-arginine, D-homoarginine, L-homoarginine, D-norarginine, L-norarginine, citrulline, a modified Arg wherein the guanidinium group is modified or substituted, Norleucine, norvaline, bhIle, Ach, N-MeArg, and N-MeIle, preferably Arg;

A7 is Cys, D-Cys, Ser, D-Ser, Ala, D-Ala, or an unnatural amino acid commonly used as a substitute thereof such as Cys(S-tBut), homoCys, Pen, (D)Pen, preferably S-tertiary butyl-cysteine, Cys(S-S-Pal), Cys(S-S-cysteamine-Pal), Cys(S-S-Cys-NHPal), and Cys(S-S-Cys);

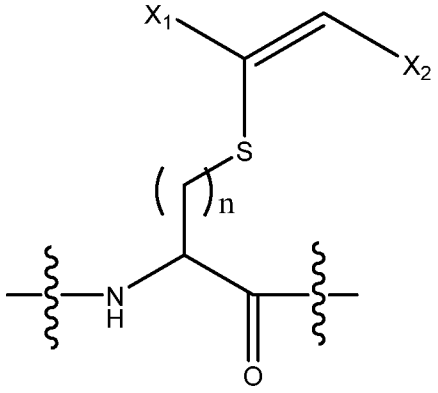
A8 is Arg, D-Arg, Ile, D-Ile, Leu, D-Leu, Thr, D-Thr, Lys, D-Lys, Val, D-Val, or an unnatural amino acid commonly used as a substitute thereof such as D-Nω,ω-dimethyl-arginine, L-Nω,ω-dimethyl-arginine, D-homoarginine, L-homoarginine, D-norarginine, L-norarginine, citrulline, a modified Arg wherein the guanidinium group is modified or substituted, Norleucine, norvaline, bhIle, Ach, N-MeArg, and N-MeIle, preferably Arg;

A9 is Phe, D-Phe, Leu, D-Leu, Ile, D-Ile, Tyr, D-Tyr, Trp, D-Trp, Phe-R^a, D-Phe-R^a, Dpa-R^a, D-Dpa-R^a, Trp-R^a, bhPhe-R^a, or an unnatural amino acid commonly used as a

substitute thereof such as PheF5, N-MePhe, benzylamide, 2-aminoindane, bhPhe, Dpa, Bip, 1Nal, 2Nal, bhDpa, and cyclohexylalanine, which may or may not have R^a linked thereto, preferably bhPhe and bhPhe-R^a, wherein R^a is palmitoyl-PEG-, wherein PEG is PEG11 or miniPEG3, palmitoyl-PEG-PEG, wherein PEG is PEG11 or miniPEG3, butanoyl (C4)-PEG11-, octanoyl (C8, Caprylic)-PEG11-, palmitoyl (C16)-PEG11-, or tetracosanoyl (C24, Lignoceric)-PEG11-; and

A10 is Cys, D-Cys, Ser, D-Ser, Ala, D-Ala, or an unnatural amino acid such as Ida, Ida(NHPal)Ahx, and Ida(NBzl2)Ahx; and

at least one of the amino acid residues A1 to A10 has the following Structural Formula A:



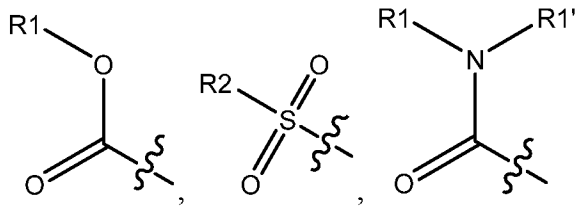
(A)

wherein

n is 1 or 2 and one or more of the hydrogens bonded to the Cn atom(s) may be substituted with a (C_1-C_3)alkyl,

X_1 and X_2 are each independently selected from the group consisting of H, alkyl, alkoxy, alkoxycarbonyl, cycloalkyl, aryl, heteroaryl, heterocycloalkyl, acyl, sulfonyl, alkylsulfonyl, alkylamino, alkylaminocarbonyl, dialkylaninocarbonyl, carboxyl, and carbamoyl;

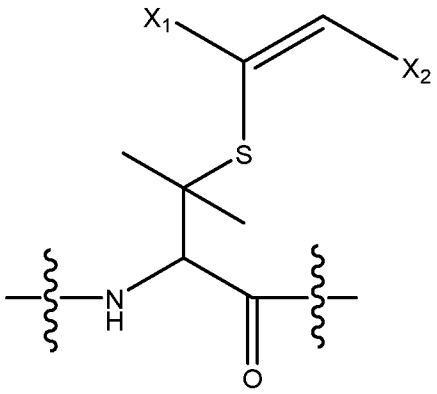
wherein the carboxy-terminal amino acid is in amide or carboxy- form; and

wherein A1, A1 to A2, A10, or a combination thereof are optionally absent. In some embodiments, X_1 and X_2, are each independently selected from the group consisting of H, phenyl, 

, and , wherein R1 and R1' are each independently selected from the group consisting of H, methyl, (C_2)alkyl, (C_3)alkyl, (C_4)alkyl, (C_1-C_5)alkyl, (C_6)alkyl, (C_7)alkyl, (C_8)alkyl, (C_9)alkyl, and (C_{10})alkyl; and R2 is –NR1R1', methyl, (C_2)alkyl, (C_3)alkyl, (C_4)alkyl, (C_1-C_5)alkyl, (C_6)alkyl, (C_7)alkyl, (C_8)alkyl, (C_9)alkyl, or (C_{10})alkyl. In some embodiments,

R1 and R1' are each independently selected from the group consisting of H, methyl, ethyl, isopropyl, and tert-butyl. In some embodiments, amino acid residue having Structural Formula A is A7.

[0039] As provided herein, "Cn atom(s)" refers to the carbon atom(s) in the parentheticals of the Structural Formulas I and A herein. Thus, an example of Structural Formula A having "one or more of the hydrogens bonded to the Cn atom(s) may be substituted with a (C_1-C_3)alkyl" is



where n is 1 and both the hydrogens are replaced with methyl.

[0040] In some embodiments, an S-alkylated hepcidin peptide according to the present invention is a hepcidin peptide having at least one amino acid residue substituted with a residue having Structural Formal A as set forth above, wherein said hepcidin peptides are selected from Table 2, Table 3, and Table 4.

[0041] In some embodiments, the amino acid residue, of the hepcidin peptides of Table 2, Table 3, or Table 4, which is substituted with a residue having Structural Formal A is the residue at amino acid position 7. In some embodiments, the amino acid residue, of the hepcidin peptides of Table 2, Table 3, or Table 4, which is substituted with a residue having Structural Formal A is a thiol containing amino acid residue.

[0042] The uncommon and unnatural amino acids referenced herein are provided in Table 5.

Table 2

Name	1	2	3	4	5	6	7	8	9	10
Hep25 DTHFPICIFCCGCCHRSKCGMCCKT (SEQ ID NO: 1)										
Hep10wt (SEQ ID NO: 2)	D	T	H	F	P	I	C	I	F	C
Length										
Hep4 (Hep4-7) (SEQ ID NO: 3)	-	-	-	F	P	I	C	-	-	-
Hep5 (Hep3-7) (SEQ ID NO: 4)	-	-	H	F	P	I	C	-	-	-
Hep6 (Hep3-8) (SEQ ID NO: 5)	-	-	H	F	P	I	C	I	-	-
Hep7ΔDT (Hep3-9) (SEQ ID NO: 6)	-	-	H	F	P	I	C	I	F	-
Hep7 (Hep1-7) (SEQ ID NO: 7)	D	T	H	F	P	I	C	-	-	-
Hep8 (Hep1-8) (SEQ ID NO: 8)	D	T	H	F	P	I	C	I	-	-
Hep9 (Hep1-9) (SEQ ID NO: 9)	D	T	H	F	P	I	C	I	F	-
Hep10 (Hep1-10 C7A) (SEQ ID NO: 10)	D	T	H	F	P	I	A	I	F	C
Thiol Modified										
Hep9F4A (SEQ ID NO: 11)	D	T	H	A	P	I	C	I	F	-
Hep9C7-SStBut (SEQ ID NO: 12)	D	T	H	A	P	I	C-S-tBut	I	F	-
Hep9C7-tBut (SEQ ID NO: 13)	D	T	H	A	P	I	C-tBut	I	F	-
Hep9-C7A (SEQ ID NO: 14)	D	T	H	F	P	I	A	I	F	-
Hep9-C7S (SEQ ID NO: 15)	D	T	H	F	P	I	S	I	F	-
(D)C (SEQ ID NO: 16)	D	T	H	F	P	I	C	I	F	-
homoC (SEQ ID NO: 17)	D	T	H	F	P	I	homoCys	I	F	-

Pen (SEQ ID NO: 18)	D	T	H	F	P	I	Pen	I	F	-
(D)Pen (SEQ ID NO: 19)	D	T	H	F	P	I	(D)Pen	I	F	-
Dap(AcBr) (SEQ ID NO: 20)	D	T	H	F	P	I	Dap(AcBr)	I	F	-
Unnatural AA's										
PR10 (SEQ ID NO: 21)	D	Tle	H	Phg	Oic	Chg	C	Chg	F	-
PR11 (SEQ ID NO: 22)	D	Tle	H	P	Oic	Chg	C	Chg	F	-
Retroinverted										
PR12 (SEQ ID NO: 23)	F	I	C	I	P	F	H	T	D	-
riHep7ΔDT (SEQ ID NO: 24)	F	I	C	I	P	F	H	-	-	-
Modified Retroinverted										
PR23 (SEQ ID NO: 25)	R2-F	I	C	I	P	F	H	T	D	-
PR24 (SEQ ID NO: 26)	R3-F	I	C	I	P	F	H	T	D	-
PR25 (SEQ ID NO: 27)	F	I	C	I	P	F	H	T	D-R6	-
PR26 (SEQ ID NO: 28)	F	I	C	I	P	F	H	T	D-R7	-
PR27 (SEQ ID NO: 29)	R4-F	I	C	I	P	F	H	T	D	-
PR28 (SEQ ID NO: 30)	R5-F	I	C	I	P	F	H	T	D	-
Modified F4 and F9										
F4bhPhe (SEQ ID NO: 31)	D	T	H	bhPhe	P	I	C	I	F	-
F4Dpa (SEQ ID NO: 32)	D	T	H	Dpa	P	I	C	I	F	-
F4Bip	D	T	H	Bip	P	I	C	I	F	-

(SEQ ID NO: 33)										
F4 1Nal (SEQ ID NO: 34)	D	T	H	1Nal	P	I	C	I	F	-
F4bhDpa (SEQ ID NO: 35)	D	T	H	bhDpa	P	I	C	I	F	-
F9bhPhe (SEQ ID NO: 36)	D	T	H	F	P	I	C	I	bhPhe	-
F9Dpa (SEQ ID NO: 37)	D	T	H	F	P	I	C	I	Dpa	-
F9Bip (SEQ ID NO: 38)	D	T	H	F	P	I	C	I	Bip	-
F91Nal (SEQ ID NO: 39)	D	T	H	F	P	I	C	I	1Nal	-
F9bhDpa (SEQ ID NO: 40)	D	T	H	F	P	I	C	I	bhDpa	-
PR39 (SEQ ID NO: 41)	D	T	H	Dpa	P	I	C	I	Dpa	-
PR40 (SEQ ID NO: 42)	D	-	Dpa	-	P	I	C	I	F	-
PR41 (SEQ ID NO: 43)	D	-	Dpa	-	P	I	C	I	Dpa	-
PR43 (SEQ ID NO: 44)	D	T	H	Dpa	P	R	C	R	Dpa	-
PR44 (SEQ ID NO: 45)	D	T	H	Dpa	Oic	I	C	I	F	-
PR45 (SEQ ID NO: 46)	D	T	H	Dpa	Oic	I	C	I	Dpa	-
PR46 (SEQ ID NO: 47)	D	T	H	Dpa	P	C	C	C	Dpa	-
Positive Charge										
PR13 (SEQ ID NO: 48)	D	T	H	F	P	I	C	I	F-R8	-
PR14 (SEQ ID NO: 49)	D	T	H	F	P	I	C	I	F-R9	-
PR15 (SEQ ID NO: 50)	D	T	H	F	P	I	C	I	F-R10	-
PR16 (SEQ ID NO: 51)	D	T	H	F	P	I	C	I	F-R11	-
PR17	D	T	H	F	P	I	C	I	F-R12	-

(SEQ ID NO: 52)										
PR18 (SEQ ID NO: 53)	D	T	H	F	P	I	C	I	F-R13	-
PR19 (SEQ ID NO: 54)	D	T	H	F	P	I	C	I	bhPhe-R8	-
PR20 (SEQ ID NO: 55)	D	T	H	F	P	I	C	I	bhPhe-R9	-
PR21 (SEQ ID NO: 56)	D	T	H	F	P	I	C	I	bhPhe-R12	-
PR22 (SEQ ID NO: 57)	D	T	H	F	P	I	C	I	bhPhe-R13	-
PR-1 (SEQ ID NO: 58)	C	Inp	(D)Dpa	Amc	R	Amc	Inp	Dpa	Cysteamide**	-
PR-2 (SEQ ID NO: 59)	C	P	(D)Dpa	Amc	R	Amc	Inp	Dpa	Cysteamide**	-
PR-3 (SEQ ID NO: 60)	C	P	(D)Dpa	Amc	R	Amc	Inp	Dpa	Cysteamide**	-
PR-4 (SEQ ID NO: 61)	C	G	(D)Dpa	Amc	R	Amc	Inp	Dpa	Cysteamide**	-

R1 = -$CONH_2$-CH_2-CH_2-S
R2 = Chenodeoxycholate-(D)Asp-(PEG11)-
R3 = Ursodeoxycholate-(D)Asp-(PEG11)-
R4 = Palmitoyl-(PEG11)-
R5 = $(Palmitoyl)_2$-Dap-PEG11- , wherein “Dap” = diaminopropionic acid
R6 = -(PEG11)-GYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO: 62)
R7 = -(PEG11)-(GPHyp)10 , “GPHyp” = Gly-Pro-hydroxyproline
R8 = -PPK
R9 = -PPR
R10 = -bhProPK
R11 = -bhProPR
R12 = -PbhProK
R13 = -PbhProR
Underlined residues = D amino acids
“-“ indicates a covalent bond, e.g. point of attachment to the given peptide
** oxidized
The PEG compound may be PEG11, i.e. O-(2-aminoethyl)-O'-(2-carboxyethyl)-undecaethyleneglycol
PR12, riHep7ΔDT, PR23, PR24, PR25, PR26, PR27 and PR28 are retroinverted mini-hepcidins and are shown, left to right, from their C-terminus to their N-terminus.

Table 3

Name	1	2	3	4	5	6	7	8	9	10
Hep10wt (SEQ ID NO: 2)	D	T	H	F	P	I	C	I	F	C
PR42' (SEQ ID NO: 63)	D	T	H	Dpa	P	R	C	R	Dpa	
PR47 (SEQ ID NO: 64)	D	T	H	Dpa	P	I	C	I	F-R4	
PR48 (SEQ ID NO: 65)	D	T	H	Dpa	P	I	C	I	Dpa-R4	
PR49 (SEQ ID NO: 66)			H	Dpa	P	I	C	I	F-R4	
PR50 (SEQ ID NO: 67)			H	Dpa	P	I	C	I	Dpa-R4	
PR51 (SEQ ID NO: 68)	D	T	H	Dpa	P	V	C	V	F-R4	
PR52 (SEQ ID NO: 69)	D	T	H	Dpa	P	L	C	L	F-R4	
PR53 (SEQ ID NO: 70)	N-MeAsp	T	H	Dpa	P	I	C	I	bhPhe-R14	
PR54 (SEQ ID NO: 71)	N-MeAsp	T	H	Dpa	bhPro	I	C	I	bhPhe-R14	
PR55 (SEQ ID NO: 72)	N-MeAsp	T	H	Dpa	P	Ach	C	Ach	F-R14	
PR56 (SEQ ID NO: 73)	N-MeAsp	T	H	Dpa	Oic	R	C	R	bhPhe-R14	
PR57 (SEQ ID NO: 74)	N-MeAsp	T	H	Dpa	bhPro	R	C	R	bhPhe-R14	
PR58 (SEQ ID NO: 75)	Ida	T	H	Dpa	P	I	C	I	bhPhe-R14	
PR59 (SEQ ID NO: 76)	Ida	T	H	Dpa	bhPro	I	C	I	bhPhe-R14	
PR60 (SEQ ID NO: 77)	Ida	T	H	Dpa	P	Ach	C	Ach	F-R14	
PR61 (SEQ ID NO: 78)	Ida	T	H	Dpa	bhPro	R	C	R	bhPhe-R14	

R4 = Palmitoyl-(PEG11)-, PEG11 = O-(2-aminoethyl)-O'-(2-carboxyethyl)-undecaethyleneglycol

R14= Palmitoyl-PEG-miniPEG3-, and “miniPEG3” = 11-amino-3,6,9-trioxaundecanoic acid
Underlined residues = D amino acids
“-“ indicates a covalent bond, e.g. point of attachment to the given peptide
In some embodiments, PEG11 can be substituted with miniPEG3 and miniPEG3 can be substituted with PEG11.

Table 4

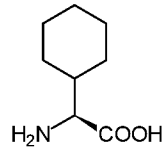
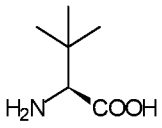
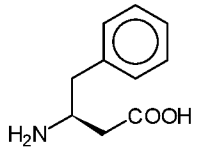
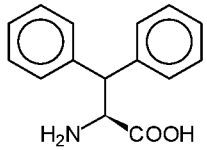
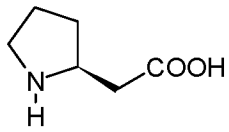
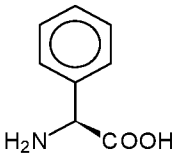
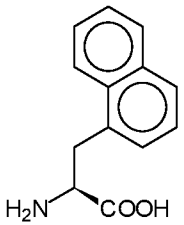
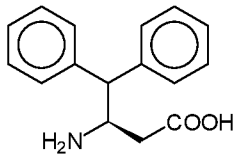
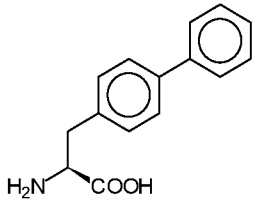
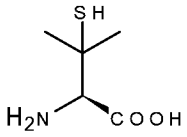
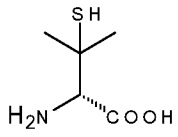
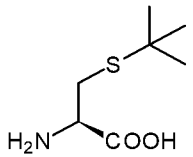
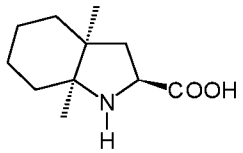
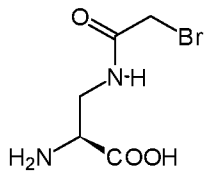
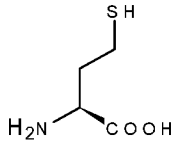
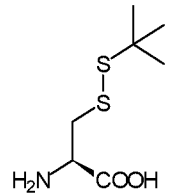
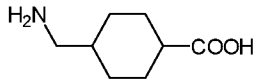
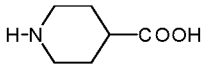
Name	1	2	3	4	5	6	7	8	9	10
Hep10wt (SEQ ID NO: 2)	D	T	H	F	P	I	C	I	F	C
PR62 (SEQ ID NO: 79)	Ida	T	H	Dpa	bhPro	R	C	R	bhPhe-R14	
PR63 (SEQ ID NO: 80)	Ida	T	H	Dpa	bhPro	N-MeArg	C	N-MeArg	bhPhe-R14	
PR64 (SEQ ID NO: 81)	Ida	T	H	Dpa	bhPro	bhArg	C	bhArg	bhPhe-R14	
PR65 (SEQ ID NO: 82)	Ida	T	H	Dpa	bhPro	R	C	R	bhPhe-R15	
PR66 (SEQ ID NO: 83)	Ida	T	H	Dpa	bhPro	R	C	R	bhPhe	
PR67 (SEQ ID NO: 84)	Ida	T	H	Dpa	bhPro	R	Cys(S-S-Pal)	R	bhPhe	
PR68 (SEQ ID NO: 85)	Ida	T	H	Dpa	bhPro	R	Cys(S-S-cysteamine-Pal)	R	bhPhe	
PR69 (SEQ ID NO: 86)	Ida	T	H	Dpa	bhPro	R	Cys(S-S-Cys-NHPal)	R	bhPhe	
PR70 (SEQ ID NO: 87)	Ida	T	H	Dpa	bhPro	R	Cys(S-S-Cys)	R	bhPhe-R14	
PR71 (SEQ ID NO: 88)	Ida(NHPal)	T	H	Dpa	bhPro	R	C	R	bhPhe	
PR72 (SEQ ID NO: 89)	Ida	T	H	Dpa	bhPro	R	C	R	bhPhe	Ida(NHPal)
PR73 (SEQ ID NO: 90)	Ida	T	H	Dpa	bhPro	R	C	R	bhPhe	Ahx-Ida(NHPal)
PR74 (SEQ ID NO: 91)	Ida	T	H	Dpa	bhPro	R	C	R	bhPhe	Ahx-Ida(NBzl2)
PR75 (SEQ ID NO: 92)	Ida	T	H	Dpa	bhPro	R	C	R	bhPhe-R16	

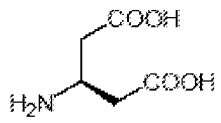
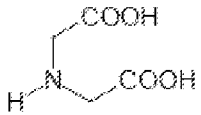
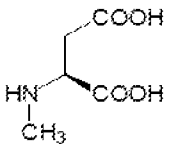
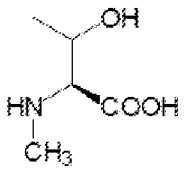
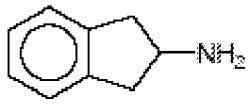
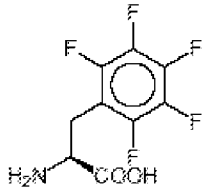
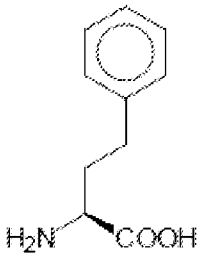
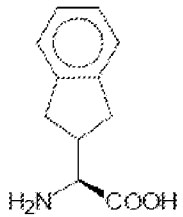
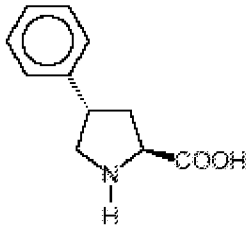
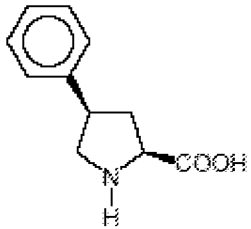
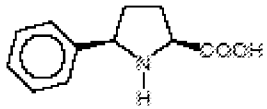
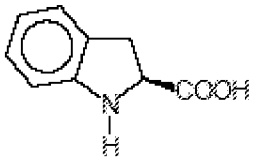
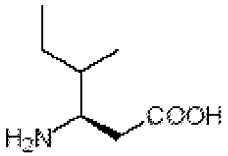
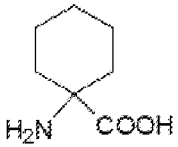
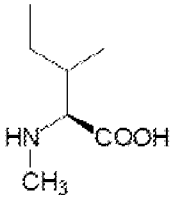
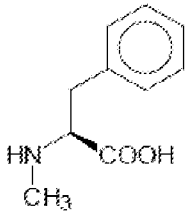
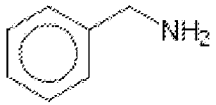
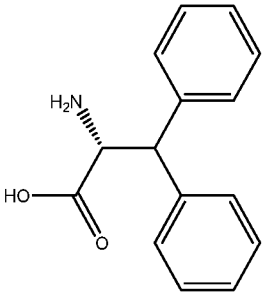
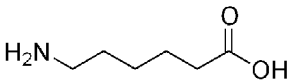
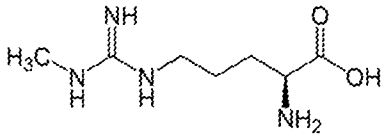
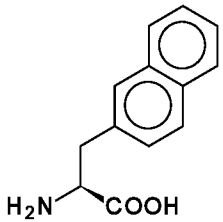
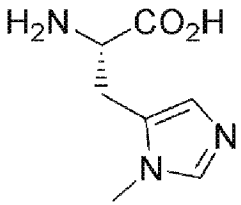
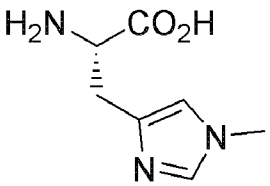
WO 2016/109363 PCT/US2015/067545

PR76 (SEQ ID NO: 93)	D	T	H	F	P	R	Cys(S-S-tBut)	R	W-R17	
PR77 (SEQ ID NO: 94)	D	T	H	F	P	R	Cys(S-S-tBut)	R	W-R18	
PR78 (SEQ ID NO: 95)	D	T	H	F	P	R	Cys(S-S-tBut)	R	W-R19	
PR79 (SEQ ID NO: 96)	D	T	H	F	P	R	Cys(S-S-tBut)	R	W-R20	
PR82 (SEQ ID NO: 97)	Ida	T	H	Dpa	bhPro	R	C	R	W	Ahx-Ida(NHPal)
PR83 (SEQ ID NO: 98)	D	T	H	F	P	R	C	R	D	
PR84 (SEQ ID NO: 99)	D	T	H	F	P	R	C	R		
PR85 (SEQ ID NO: 100)	D	T	H	F	P	R	C	R	D	
PR86 (SEQ ID NO: 101)	D	T	H	F	P	R	C	R		

R4 = Palmitoyl-(PEG11)-, wherein PEG11 = O-(2-aminoethyl)-O'-(2-carboxyethyl)-undecaethyleneglycol
R14 = Palmitoyl-PEG-miniPEG3-, and "miniPEG3" = 11-amino-3,6,9-trioxaundecanoic acid
R15 = Palmitoyl-PEG-
R16 = C16
R17 = Butanoyl-PEG11-
R18 = Octanoyl-PEG11-
R19 = Palmitoyl-PEG11-
R20 = Tetracosanoyl-PEG11-
Ahx-Ida(NHPal) = Aminohexanoic acid spacer between peptide residue 9 and Ida residue; Palmitylamine amide on Ida side chain
Ida(NHPal) = Palmitylamine amide on Ida side chain
Ida(NBzl2) = N,N'-Dibenzylamine amide on Ida side chain
Cys(S-S-Pal) = Palmitoyl attached to Cys7 via a disufide bond
Cys(S-S-cysteamine-Pal) = Palmitoyl attached to Cys7 via SS-Cysteamine
Cys(S-S-Cys-NHPal) = Palmitylamine attached to Cys7 via another Cys
Cys(S-S-Cys) = Cys attached to Cys7 via disulfide bond
Underlined residues = D amino acids
"-" indicates a covalent bond, e.g. point of attachment to the given peptide
In some embodiments, PEG11 can be substituted with miniPEG3.
In some embodiments, miniPEG3 can be substituted with PEG11.
In some embodiments, PEG can be substituted with PEG11, but not miniPEG3.

TABLE 5
Uncommon or Unnatural Amino Acids

Chg	**Tle**	**bhPhe**	**Dpa**	**bhPro**	**Phg**
					
L-α-cyclohexylglycine	L-tert-leucine	β-homophenylalanine	3,3-diphenyl-L-alanine	L-beta-homoproline	L-phenylglycine
1Nal	**bhDpa**	**Bip**	**Pen**	**(D)Pen**	**Cys(tBut)**
					
(1-naphthyl)-L-alanine	(S)-3-Amino-4,4-diphenylbutanoic acid	L-biphenylalanine	L-Penicillamine	D-Penicillamine	S-t-butyl-L-cysteine
Oic	**Dap(AcBr)**	**homoCys**	**Cys(S-tBut)**	**Amc**	**Inp**
					
octahydroindole-2-carboxylic acid	N^{γ}-(bromoacetyl)-L-2,3-diaminopropionic acid	L-homocysteine	S-t-Butylthio-L-cysteine	4-(aminomethyl)cyclohexane carboxylic acid	isonipecotic acid
bhAsp	**Ida**	**N-MeAsp**	**N-MeThr**	**2-Aminoindane**	**PheF5**

					
hPhe	**hgl**	**trans-4-PhPro**	**cis-4-PhPro**	**cis-5-PhPro**	**Idc**
					
bhIle	**Ach**	**N-MeIle**	**N-MePhe**	**Benzylamide**	**(D)Dpa**
					
					3,3-diphenyl-D-alanine
Ahx	**N-MeArg**		**2Nal**	**L-His(π-Me)**	**L-His(τ-Me)**
					

[0043] As provided herein, a bond is represented by a line, such as "–", or the symbol "〰". The line and symbol represent that the bond is the point of attachment between two molecular subunits. As used herein, usage of "(C_n-C_m)" indicates the range of carbon atoms the indicated hydrocarbon may have. For example, the term "(C_1-C_6)alkyl" refers to a straight or branched hydrocarbon from 1 to 6 carbon atoms and includes methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tert-butyl, n-pentyl, n-hexyl, and the like. Similarly, usage of "(C_n)" indicates the number of carbon atoms the indicated hydrocarbon contains.

[0044] An "alkyl" refers to a straight or branched chain monovalent radical of saturated and/or unsaturated carbon atoms and hydrogen atoms, such as methyl (Me) ethyl (Et) propyl (Pr) isopropyl (i-Pr) butyl (n-Bu) isobutyl (i-Bu) t-butyl (t-Bu) (sec-Bu) ethenyl, pentenyl, butenyl, propenyl, ethynyl, butynyl, propynyl, pentynyl, hexynyl, and the like, which may be unsubstituted (i.e., contain only carbon and hydrogen) or substituted by one or more substituents as defined below. The term "(C_1-C_6)alkyl" as used herein refers to a straight or branched hydrocarbon from 1 to 6 carbon atoms and includes methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tert-butyl, n-pentyl, n-hexyl, and the like. The (C_1-C_6)alkyl group optionally can be substituted with one or more substituents as defined below. The term "(C_1-C_3)alkyl" as used herein refers to a straight or branched hydrocarbon of from 1 to 3 carbon atoms and includes methyl, ethyl, n-propyl, isopropyl, and the like. The (C_1-C_3)alkyl group optionally can be substituted with one or more of more substituents as defined below.

[0045] An "alkoxy" refers to the radical –OR, where R is a straight or branched chain alkyl group. Exemplary alkoxy groups include methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, pentoxy, and the like. A "(C_1-C_6)alkoxy" refers to a straight or branched chain alkoxy group containing from 1 to 6 carbon atoms and a "(C_1-C_3)alkoxy" refers to a straight or branched chain alkoxy group containing from 1 to 3 carbon atoms.

[0046] An "alkoxycarbonyl" refers to the radical –C(O)OR, where R is an alkyl group.

[0047] A "cycloalkyl" refers to a non-aromatic monovalent monocyclic, bicyclic, or tricyclic radical comprising 3-14 carbon ring atoms, each of which may be saturated or unsaturated, and which may be unsubstituted or substituted by one or more suitable substituents as defined below, and to which may be fused one or more heterocycloalkyl groups, aryl groups, or heteroaryl groups, which themselves may be unsubstituted or substituted by one or more substituents. The term "(C_3-C_8)cycloalkyl" means a hydrocarbon ring containing from 3 to 8 carbon atoms, for example, cyclopropyl,

cyclobutyl, cyclopentyl, or cyclohexyl. Where possible, the cycloalkyl group may contain double bonds, for example, 3-cyclohexen-1-yl. The cycloalkyl ring may be unsubstituted or optionally may be substituted by one or more substituents selected from (C_1-C_6)alkyl, (C_1-C_6)alkoxy, (C_1-C_6)thioalkoxy, hydroxy, thiol, halo, formyl, carboxyl, amino, aminoalkyl, –CO_2(C_1-C_6)alkyl, –CO(C_1-C_6)alkyl, –C(O)N(C_1-C_6)alkyl, aryl, and heteroaryl.

[0048] An "aryl" refers to a cyclic or polycyclic aromatic ring having from 5 to 12 carbon atoms, and may be unsubstituted or substituted by one or more suitable substituents as defined below, and to which may be fused one or more cycloalkyl groups, heterocycloalkyl groups, or heteroaryl groups, which themselves may be unsubstituted or substituted by one or more suitable substituents.

[0049] A "heteroaryl" refers to an aromatic monovalent monocyclic, bicyclic, or tricyclic radical comprising 4-18 ring members, including 1-5 heteroatoms selected from nitrogen, oxygen, and sulfur, which may be unsubstituted or substituted by one or more suitable substituents as defined below, and to which may be fused one or more cycloalkyl groups, heterocycloalkyl groups, or aryl groups, which themselves may be unsubstituted or substituted by one or more suitable substituents.

[0050] A "heterocycloalkyl" refers to a non-aromatic monovalent monocyclic, bicyclic, or tricyclic radical, which is saturated or unsaturated, comprising 3-18 ring members, which includes 1-5 heteroatoms selected from nitrogen, oxygen, and sulfur, where the radical is unsubstituted or substituted by one or more suitable substituents as defined below, and to which may be fused one or more cycloalkyl groups, aryl groups, or heteroaryl groups, which themselves may be unsubstituted or substituted by one or more suitable substituents.

[0051] An "acyl" refers to a –C(O)-R radical, where R is a suitable substituent as defined below.

[0052] A "sulfonyl" refers to a –SO_2R radical, where R is a suitable substituent as defined below.

[0053] An "alkylsulfonyl" refers to the radical –SO_2R, where R is an alkyl group.

[0054] An "alkylamino" refers to an amino moiety substituted with one (i.e., –NHR) or two (i.e., –NRR') (C_1-C_6)alkyl groups which may be the same or different. Examples of such alkylamino groups include aminomethyl, dimethylamino, aminomethylethyl, aminomethylpropyl, and the like.

[0055] An "alkylaminocarbonyl" refers to the radical –C(O)NHR, where R is an alkyl group.

[0056] A "dialkylaminocarbonyl" refers to the radical –C(O)NRR', where each R may be the same or different alkyl group.

[0057] A "carboxyl" refers to the radical –C(O)OH.

[0058] A "carbamoyl group" refers to the radical $C(O)NH_2$.

[0059] In general, the various moieties or functional groups for variables in the formulae may be "optionally substituted" by one or more suitable "substituents". The term "substituent" or "suitable substituent" refers to any suitable substituent that may be recognized or selected, such as through routine testing, by those skilled in the art. In some embodiments, the substituent is N, O, Si, P, or S.

[0060] As used herein, a "disease of iron metabolism" includes diseases where aberrant iron metabolism directly causes the disease, or where iron blood levels are dysregulated causing disease, or where iron dysregulation is a consequence of another disease, or where diseases can be treated by modulating iron levels, and the like. More specifically, a disease of iron metabolism according to this disclosure includes iron overload diseases, iron deficiency disorders, disorders of iron biodistribution, other disorders of iron metabolism and other disorders potentially related to iron metabolism, etc. Diseases of iron metabolism include hemochromatosis, HFE mutation hemochromatosis, ferroportin mutation hemochromatosis, transferrin receptor 2 mutation hemochromatosis, hemojuvelin mutation hemochromatosis, hepcidin mutation hemochromatosis, juvenile hemochromatosis, neonatal hemochromatosis, hepcidin deficiency, transfusional iron overload, thalassemia, thalassemia intermedia, alpha thalassemia, sideroblastic anemia, polycythemia vera, myelodysplastic syndromes, porphyria, porphyria cutanea tarda, African iron overload, hyperferritinemia, ceruloplasmin deficiency, atransferrinemia, congenital dyserythropoietic anemia, anemia of chronic disease, anemia of inflammation, anemia of infection, hypochromic microcytic anemia, iron-deficiency anemia, iron-refractory iron deficiency anemia, anemia of chronic kidney disease, erythropoietin resistance, iron deficiency of obesity, other anemias, benign or malignant tumors that overproduce hepcidin or induce its overproduction, conditions with hepcidin excess, Friedreich ataxia, gracile syndrome, Hallervorden-Spatz disease, Wilson's disease, pulmonary hemosiderosis, hepatocellular carcinoma, cancer, hepatitis, cirrhosis of liver, pica, chronic renal failure, insulin resistance, diabetes, atherosclerosis, neurodegenerative disorders, multiple sclerosis, Parkinson's disease, Huntington's disease, and Alzheimer's disease. As used herein, "iron overload diseases" and "diseases of iron overload" refer diseases and disorders that result in or may cause abnormally high levels of iron in afflicted subjects if untreated.

[0061] In some cases the diseases and disorders included in the definition of "disease of iron metabolism" are not typically identified as being iron related. For example, hepcidin is highly expressed in the murine pancreas suggesting that diabetes (Type I or Type II), insulin resistance, glucose intolerance, and other disorders may be ameliorated by treating underlying iron metabolism disorders. See Ilyin, G. et al. (2003) FEBS Lett. 542 22-26, which is herein incorporated by reference. As such, these diseases are encompassed under the broad definition. Those skilled in the art are readily able to determine whether a given disease is a "disease or iron metabolism" according to the present invention using methods known in the art, including the assays of WO 2004092405, which is herein incorporated by reference, and assays which monitor hepcidin, hemojuvelin, or iron levels and expression, which are known in the art such as those described in U.S. Patent No. 7,534,764, which is herein incorporated by reference. In some embodiments of the present invention, the diseases of iron metabolism are iron overload diseases, which include hereditary hemochromatosis, iron-loading anemias, alcoholic liver diseases and chronic hepatitis C.

[0062] As used herein, a compound having "hepcidin activity" means that the compound has the ability to lower plasma iron concentrations in subjects (e.g. mice or humans), when administered thereto (e.g. parenterally injected or orally administered), in a dose-dependent and time-dependent manner. See e.g. as demonstrated in Rivera et al. (2005), Blood 106:2196-9.

[0063] In some embodiments, the peptides of the present invention have *in vitro* activity as assayed by the ability to cause the internalization and degradation of ferroportin in a ferroportin-expressing cell line as taught in Nemeth et al. (2006) Blood 107:328-33. *In vitro* activity may be measured by the dose-dependent loss of fluorescence of cells engineered to display ferroportin fused to green fluorescent protein as in Nemeth et al. (2006) Blood 107:328-33. Aliquots of cells are incubated for 24 hours with graded concentrations of a reference preparation of Hep25 or the S-alkylated hepcidin peptide to be tested. As provided herein, the EC_{50} values are provided as the concentration of a given compound (e.g. peptide) that elicits 50% of the maximal loss of fluorescence generated by the reference Hep25 preparation. EC_{50} of Hep25 preparations in this assay range from 5 to 15 nM and some preferred S-alkylated hepcidin peptides have EC_{50} values in *in vitro* activity assays of about 1,000 nM or less.

[0064] Other methods known in the art for calculating the hepcidin activity and *in vitro* activity of peptides according to the present invention may be used. For example, the *in vitro* activity of compounds may be measured by their ability to internalize cellular

ferroportin, which is determined by immunohistochemistry or flow cytometry using antibodies which recognizes extracellular epitopes of ferroportin. Alternatively, the *in vitro* activity of compounds may be measured by their dose-dependent ability to inhibit the efflux of iron from ferroportin-expressing cells that are preloaded with radioisotopes or stable isotopes of iron, as in Nemeth et al. (2006) Blood 107:328-33.

[0065] One or more S-alkylated hepcidin peptides according to the present invention, alone or in combination with one or more mini-hepcidins and/or one or more modified mini-hepcidins, may be administered to subjects in order to treat, e.g., inhibit and/or reduce, iron overload in subjects, such as humans. Therefore, S-alkylated hepcidin peptides according to the present invention may be used in medicaments and treatments in order to treat iron overload disorders, e.g. beta-thalassemia and hereditary hemochromatosis, by inhibiting and/or reducing iron overload in subjects. In some embodiments, at least one S-alkylated hepcidin peptide is administered to a subject before, during, after, or a combination thereof, symptoms of iron overload are observed and/or being diagnosed as having an iron overload disorder.

[0066] In some embodiments, one or more S-alkylated hepcidin peptides, alone or in combination with one or more mini-hepcidins and/or modified mini-hepcidins, are provided in the form of a composition which comprises a carrier suitable for its intended purpose. The compositions may also include one or more additional ingredients suitable for its intended purpose. For example, for assays, the compositions may comprise liposomes, niclosamide, SL220 solubilization agent (NOF, Japan), cremophor EL (Sigma), ethanol, and DMSO. For treatment of an iron overload disease, the compositions may comprise different absorption enhancers and protease inhibitors, solid microparticles or nanoparticles for peptide encapsulation (such as chitosan and hydrogels), macromolecular conjugation, lipidization and other chemical modification.

[0067] The present invention also provides kits comprising one or more S-alkylated hepcidin peptides, alone or in combination with one or more mini-hepcidins, one or more modified mini-hepcidins, and/or compositions of the present invention packaged together with reagents, devices, instructional material, or a combination thereof. For example, the kits may include reagents used for conducting assays, drugs, and compositions for diagnosing, treating, or monitoring disorders of iron metabolism, devices for obtaining samples to be assayed, devices for mixing reagents and conducting assays, and the like.

[0068] As the S-alkylated hepcidin peptides of the present invention exhibit hepcidin activity, i.e., act as agonists of ferroportin degradation, one or more S-alkylated hepcidin peptides, alone or in combination with one or more mini-hepcidins and/or modified mini-

hepcidins, may be used to treat iron overload diseases. For example, one or more S-alkylated hepcidin peptides, alone or in combination with one or more mini-hepcidins and/or modified mini-hepcidins, may be administered to a subject to ameliorate the symptoms and/or pathology associated with iron overload in iron-loading anemias (especially β-thalassemias) where phlebotomy is contraindicated and iron chelators are the mainstay of treatment but are often poorly tolerated. One or more S-alkylated hepcidin peptides, alone or in combination with one or more mini-hepcidins and/or modified mini-hepcidins, may be used to treat hereditary hemochromatosis, especially in subjects who do not tolerate maintenance phlebotomy. One or more S-alkylated hepcidin peptides, alone or in combination with one or more mini-hepcidins and/or modified mini-hepcidins, may be used to treat acute iron toxicity. In some embodiments, treatment with one or more S-alkylated hepcidin peptides, alone or in combination with one or more mini-hepcidins and/or modified mini-hepcidins, may be combined with phlebotomy or chelation.

[0069] One or more S-alkylated hepcidin peptides, alone or in combination with one or more mini-hepcidins and/or modified mini-hepcidins, may be administered to a subject, preferably a mammal such as a human. In some embodiments, the administration to the subject is before, during, and/or after the subject exhibits an increase in iron levels and/or abnormally high levels of iron. In some embodiments, the subject to be treated is one who is at risk of having high levels of iron and/or has a genetic predisposition to having an iron overload disease. In some embodiments, the peptides are administered in a form of a pharmaceutical composition. In some embodiments, the peptides are administered in a therapeutically effective amount. As used herein, a "therapeutically effective amount" is an amount which ameliorates the symptoms and/or pathology of a given disease of iron metabolism as compared to a control such as a placebo.

[0070] A therapeutically effective amount may be readily determined by standard methods known in the art. The dosages to be administered can be determined by one of ordinary skill in the art depending on the clinical severity of the disease, the age and weight of the subject, or the exposure of the subject to iron. In some embodiments, therapeutically effective amounts of S-alkylated hepcidin peptides range from about 0.01 to about 10 mg/kg body weight, about 0.01 to about 3 mg/kg body weight, about 0.01 to about 2 mg/kg, about 0.01 to about 1 mg/kg, or about 0.01 to about 0.5 mg/kg body weight for parenteral formulations. In some embodiments, therapeutically effective amounts for oral administration may be up to about 10-fold higher. Moreover, treatment of a subject with a peptide or composition of the present invention can include a single treatment or, preferably, can include a series of treatments. It will be appreciated that the actual dosages

will vary according to the particular peptide or composition, the particular formulation, the mode of administration, and the particular site, host, and disease being treated. It will also be appreciated that the effective dosage used for treatment may increase or decrease over the course of a particular treatment. Optimal dosages for a given set of conditions may be ascertained by those skilled in the art using conventional dosage-determination tests in view of the experimental data for a given peptide or composition. Changes in dosage may result and become apparent by standard diagnostic assays known in the art. In some conditions chronic administration may be required.

[0071] The pharmaceutical compositions of the invention may be prepared in a unit-dosage form appropriate for the desired mode of administration. The compositions of the present invention may be administered for therapy by any suitable route including oral, rectal, nasal, topical (including buccal and sublingual), vaginal and parenteral (including subcutaneous, intramuscular, intravenous and intradermal). A variety of administration routes can be used in accordance with the present invention, including oral, topical, transdermal, nasal, pulmonary, transpercutaneous (wherein the skin has been broken either by mechanical or energy means), rectal, buccal, vaginal, via an implanted reservoir, or parenteral. Parenteral includes subcutaneous, intravenous, intramuscular, intraperitoneal, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional, and intracranial injection or infusion techniques, as well as injectable materials (including polymers) for localized therapy. In some embodiments, the route of administration is subcutaneous. In some embodiments, the composition is in a sealed sterile glass vial. In some embodiments, the composition contains a preservative. Pharmaceutical compositions may be formulated as bulk powder, tablets, liquids, gels, lyophilized, and the like, and may be further processed for administration. See e.g., REMINGTON: THE SCIENCE AND PRACTICE OF PHARMACY. 20th ed. (2000) Lippincott Williams & Wilkins. Baltimore, MD, and subsequent editions.

[0072] It will be appreciated that the preferred route will vary with the condition and age of the recipient, the nature of the condition to be treated, and the chosen peptide and composition. Pharmaceutical compositions of the present invention comprise a therapeutically effective amount of at least one peptide as disclosed herein, and a pharmaceutically acceptable carrier or diluent, which may be inert. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, bulking agent, coatings, antibacterial and antifungal agents, preservatives, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration and known in the art. Except insofar as any conventional

media or agent is incompatible with the active compound, use thereof in the compositions is contemplated.

[0073] Supplementary compounds can also be incorporated into the compositions. Supplementary compounds include niclosamide, liposomes, SL220 solubilization agent (NOF, Japan), Cremophor EL (Sigma), ethanol, and DMSO.

[0074] Toxicity and therapeutic efficacy of the peptides and compositions of the present invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Peptides which exhibit large therapeutic indices are preferred. While peptides that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such peptides to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[0075] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of peptides of the present invention lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any peptide used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography or by mass spectroscopy.

[0076] The resulting decrease of plasma iron could also reduce the levels of toxic non-transferrin bound iron (NTBI) and promote the mobilization of iron from the heart and endocrine organs where iron excess is not tolerated. Thus, in some embodiments, one or more S-alkylated hepcidin peptides may be administered to a subject in order to reduce the levels of NTBI and/or promote the mobilization of iron from the heart and endocrine organs to other organs and tissues. In some embodiments, in established iron overload in human subjects, effective treatment with one or more S-alkylated hepcidin peptides may include more than one dose per day, a prolonged treatment period before a beneficial

effect in liver iron can be detected, or may be combined with removal of iron by phlebotomy or chelation.

[0077] According to U.S. Food and Drug Administration dosing adjustment guidelines, the difference in metabolic rates between the mouse and human requires a conversion based on the Km factor which normalizes doses to body surface area (Reagan-Shaw S, et al. (2008) FASEB J 22(3):659-661). A human equivalent dose (HED) can be estimated by HED = animal dose (mg/kg) x (animal Km/human Km), where the Km for mouse and an adult human are 3 and 37, respectively. Thus, according to the present invention, a subcutaneous dose of an S-alkylated hepcidin peptide in a human could be up to about 50-100 μg/kg/d, about 75-125 μg/kg/d, or about 90-110 μg/kg/d, preferably about 100 μg/kg/d (as this dose is a readily administrable amount of peptide about three times the median basal dose of the most widely used peptide drug, subcutaneous insulin, commonly used at 0.75 U/kg/d or 33 μg/kg/d in type 2 diabetics (Rosenstock J, et al. (2001) Diabetes Care 24(4):631-636)). Of course, lower doses, as well as higher doses, depending on the particular mini-hepcidin, form of administration, formulation, the subject, and the degree of iron overload, may be administered to subject. In some embodiments, a therapeutically effective dose of one or more S-alkylated hepcidin peptides ranges from about 10-500 μg/kg/d. Again, lower doses, as well as higher doses, depending on the particular mini-hepcidin, form of administration, formulation, the subject, and the degree of iron overload, may be administered to subject.

[0078] As provided herein, S-alkylated hepcidin peptides according to the present invention may be used to inhibit, reduce, or treat iron overload in subjects at risk due to genetic defects or those who have already undergone iron depletion, but no longer tolerate chelation or venesection therapy. The S-alkylated hepcidin peptides according to the present invention may be used to treat a subject having β-thalassemia major and/or a subject having hepcidin levels that are higher than normal but are lower than what is appropriate for the degree of iron overload and the particular subject. For example, one or more S-alkylated hepcidin peptides according to the present invention may be used to treat a subject who suffers from hyperabsorption of dietary iron, but has normal levels of iron, in order to lower the amount of iron in the subject and offset the hyperabsorption. One or more S-alkylated hepcidin peptides according to the present invention may be used to treat ineffective erythropoiesis and improve anemia in subjects.

[0079] Because of the relatively small size of the S-alkylated hepcidin peptides of the present invention, the S-alkylated hepcidin peptides may be appropriately formulated and optimized for oral administration or administration by other noninvasive means such as

those used for insulin administration (Roach P. (2008) Clinical Pharmacokinetics 47(9):595-610) such as inhalation, or transcutaneous delivery, or mucosal nasal or buccal delivery.

[0080] PR73SH appears to be remarkably stable in mildly oxidizing conditions as prolonged storage of the compound in DMSO (10 mM solution) at room temperature for 30 days shows very limited levels of decomposition or sulfide oxidation (99.5 ± 0.5% of stability, determined by LC/MS/MS experiments). Thus, the present invention also provides storage stable compositions comprising one or more S-alkylated hepcidin peptides.

[0081] Section headings are used for organizational purposes only and are not to be construed as defining or limiting the subject matter described. Unless explicitly provided otherwise, singular word forms include the plural forms. As used herein, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. As used herein, "and/or" means "and" or "or". For example, "A and/or B" means "A, B, or both A and B" and "A, B, and/or C" means "A, B, C, or a combination thereof" and said "combination thereof" means "A and B, A and C, or B and C". As used herein, "or" can mean "and/or" unless stated otherwise or the context clearly dictates otherwise.

[0082] In the event of a discrepancy between the sequences set forth in the sequence listing and the Tables, the sequences in the Table are controlling.

[0083] To the extent necessary to understand or complete the disclosure of the present invention, all publications, patents, and patent applications mentioned herein are expressly incorporated by reference therein to the same extent as though each were individually so incorporated.

[0084] Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations, and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

WHAT IS CLAIMED IS:

1. An S-alkylated hepcidin peptide comprising or consisting of the following Structural Formula IA or IB

A1-A2-A3-A4-A5-A6-A7-A8-A9-A10 IA

A10-A9-A8-A7-A6-A5-A4-A3-A2-A1 IB

wherein

A1 is Asp, D-Asp, Glu, D-Glu, pyroglutamate, D-pyroglutamate, Gln, D-Gln, Asn, D-Asn, or an unnatural amino acid commonly used as a substitute thereof such as bhAsp, Ida, Ida(NHPal), and N-MeAsp, preferably Ida and N-MeAsp;

A2 is Thr, D-Thr, Ser, D-Ser, Val, D-Val, Ile, D-Ile, Ala, D-Ala or an unnatural amino acid commonly used as a substitute thereof such as Tle, Inp, Chg, bhThr, and N-MeThr;

A3 is His, D-His, Asn, D-Asn, Arg, D-Arg, or an unnatural amino acid commonly used as a substitute thereof such as L-His(π-Me), D-His(π-Me), L-His(τ-Me), or D-His(τ-Me);

A4 is Phe, D-Phe, Leu, D-Leu, Ile, D-Ile, Trp, D-Trp, Tyr, D-Tyr, or an unnatural amino acid commonly used as a substitute thereof such as Phg, bhPhe, Dpa, Bip, 1Nal, 2Nal, bhDpa, Amc, PheF5, hPhe, Igl, or cyclohexylalanine, preferably Dpa;

A5 is Pro, D-Pro, Ser, D-Ser, or an unnatural amino acid commonly used as a substitute thereof such as Oic, bhPro, trans-4-PhPro, cis-4-PhPro, cis-5-PhPro, and Idc, preferably bhPro;

A6 is Arg, D-Arg, Ile, D-Ile, Leu, D-Leu, Thr, D-Thr, Lys, D-Lys, Val, D-Val, or an unnatural amino acid commonly used as a substitute thereof such as D-Nω,ω-dimethyl-arginine, L-Nω,ω-dimethyl-arginine, D-homoarginine, L-homoarginine, D-norarginine, L-norarginine, citrulline, a modified Arg wherein the guanidinium group is modified or substituted, Norleucine, norvaline, bhIle, Ach, N-MeArg, and N-MeIle, preferably Arg;

A7 is Cys, D-Cys, Ser, D-Ser, Ala, D-Ala, or an unnatural amino acid commonly used as a substitute thereof such as Cys(S-tBut), homoCys, Pen, (D)Pen, preferably S-tertiary butyl-cysteine, Cys(S-S-Pal), Cys(S-S-cysteamine-Pal), Cys(S-S-Cys-NHPal), and Cys(S-S-Cys);

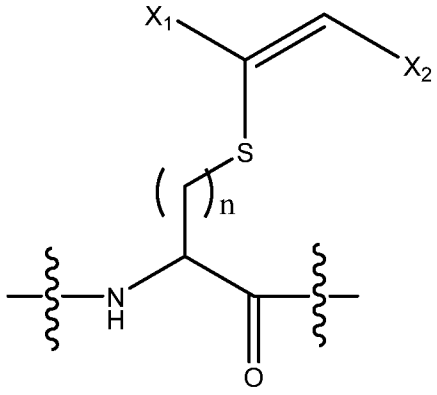
A8 is Arg, D-Arg, Ile, D-Ile, Leu, D-Leu, Thr, D-Thr, Lys, D-Lys, Val, D-Val, or an unnatural amino acid commonly used as a substitute thereof such as D-Nω,ω-dimethyl-arginine, L-Nω,ω-dimethyl-arginine, D-homoarginine, L-homoarginine,

D-norarginine, L-norarginine, citrulline, a modified Arg wherein the guanidinium group is modified or substituted, Norleucine, norvaline, bhIle, Ach, N-MeArg, and N-MeIle, preferably Arg;

A9 is Phe, D-Phe, Leu, D-Leu, Ile, D-Ile, Tyr, D-Tyr, Trp, D-Trp, Phe-R^a, D-Phe-R^a, Dpa-R^a, D-Dpa-R^a, Trp-R^a, bhPhe-R^a, or an unnatural amino acid commonly used as a substitute thereof such as PheF5, N-MePhe, benzylamide, 2-aminoindane, bhPhe, Dpa, Bip, 1Nal, 2Nal, bhDpa, and cyclohexylalanine, which may or may not have R^a linked thereto, preferably bhPhe and bhPhe-R^a, wherein R^a is palmitoyl-PEG-, wherein PEG is PEG11 or miniPEG3, palmitoyl-PEG-PEG, wherein PEG is PEG11 or miniPEG3, butanoyl (C4)-PEG11-, octanoyl (C8, Caprylic)-PEG11-, palmitoyl (C16)-PEG11-, or tetracosanoyl (C24, Lignoceric)-PEG11-; and

A10 is Cys, D-Cys, Ser, D-Ser, Ala, D-Ala, or an unnatural amino acid such as Ida, Ida(NHPal)Ahx, and Ida(NBzl2)Ahx; and

at least one of the amino acid residues A1 to A10 has Structural Formula A:



(A)

wherein

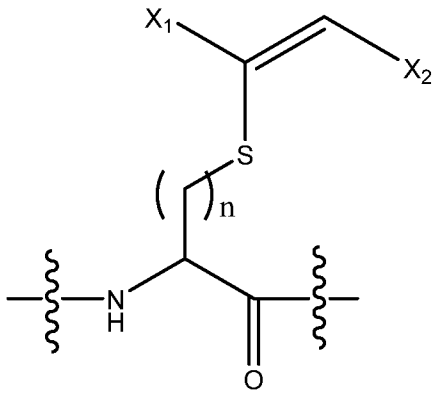
n is 1 or 2 and one or more of the hydrogens bonded to the Cn atom(s) may be substituted with a (C_1-C_3)alkyl,

X_1 and X_2 are each independently selected from the group consisting of H, alkyl, alkoxy, alkoxycarbonyl, cycloalkyl, aryl, heteroaryl, heterocycloalkyl, acyl, sulfonyl, alkylsulfonyl, alkylamino, alkylaminocarbonyl, dialkylaninocarbonyl, carboxyl, and carbamoyl;

wherein the carboxy-terminal amino acid is in amide or carboxy- form; and

wherein A1, A1 to A2, A10, or a combination thereof are optionally absent.

2. An S-alkylated hepcidin peptide comprising or consisting of an amino acid sequence selected from SEQ ID NOs: 1-101 with at least one amino acid substitution, said at least one amino acid substitution has the Structural Formula



(A)

wherein

n is 1 or 2 and one or more of the hydrogens bonded to the Cn atom(s) may be substituted with a (C_1-C_3)alkyl,

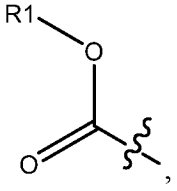
X_1 and X_2 are each independently selected from the group consisting of H, alkyl, alkoxy, alkoxycarbonyl, cycloalkyl, aryl, heteroaryl, heterocycloalkyl, acyl, sulfonyl, alkylsulfonyl, alkylamino, alkylaminocarbonyl, dialkylaninocarbonyl, carboxyl, and carbamoyl;

wherein the carboxy-terminal amino acid is in amide or carboxy- form.

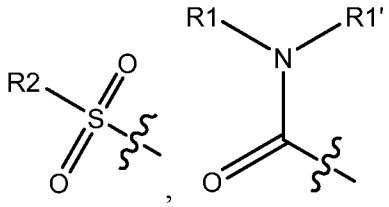
3. The S-alkylated hepcidin peptide according to claim 1, wherein the amino acid residue having Structural Formula A is A7.

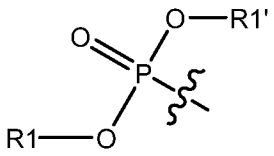
4. The S-alkylated hepcidin peptide of claim 3, wherein A1 is Ida, A2 is Thr, A3 is His, A4 is Dpa, A5 is bhPro, A6 is Arg, A8 is Arg, A9 is bhPhe, and A10 is Ahx-Ida(NHPal).

5. The S-alkylated hepcidin peptide according to claim 2, wherein the amino acid residue having Structural Formula A corresponds to a thiol containing amino acid of SEQ ID NOs: 1-101.

6. The S-alkylated hepcidin peptide according to any one of claims 1 to 5, wherein X_1 and X_2, are each independently selected from the group consisting of H, phenyl, 

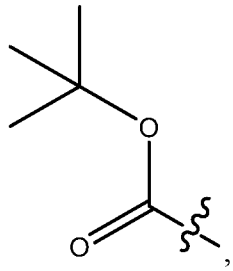
,



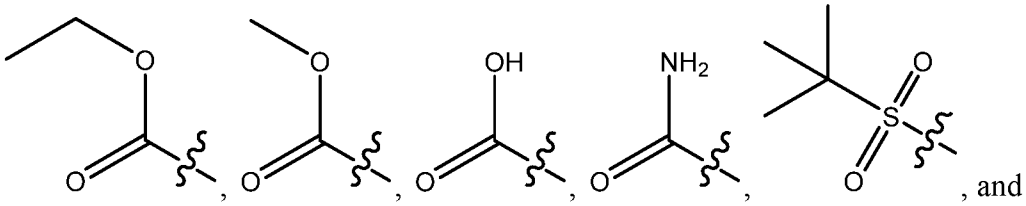
, and 

, wherein R1 and R1' are each independently selected from the group consisting of H, methyl, (C_2)alkyl, (C_3)alkyl, (C_4)alkyl, (C_1-C_5)alkyl, (C_6)alkyl, (C_7)alkyl, (C_8)alkyl, (C_9)alkyl, and (C_{10})alkyl; and R2 is –NR1R1', methyl, (C_2)alkyl, (C_3)alkyl, (C_4)alkyl, (C_1-C_5)alkyl, (C_6)alkyl, (C_7)alkyl, (C_8)alkyl, (C_9)alkyl, and (C_{10})alkyl.

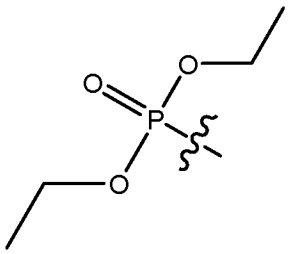
7. The S-alkylated hepcidin peptide according to claim 6, wherein R1 and R1' are each independently selected from the group consisting of H, methyl, ethyl, isopropyl, and tert-butyl.

8. The S-alkylated hepcidin peptide according to any one of claims 1 to 5, wherein X_1 and X_2 are each independently selected from the group consisting of H, phenyl, 

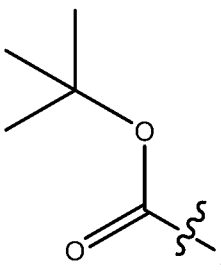
,

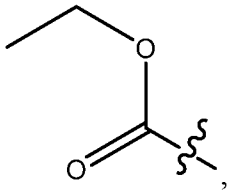


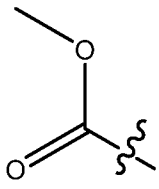
, and



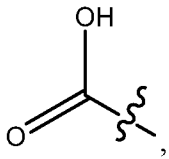
.

9. The S-alkylated hepcidin peptide according to any one of claims 1 to 5, wherein X_1 and X_2 are (a) both 

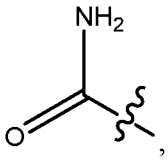
, (b) both 

, (c) both 

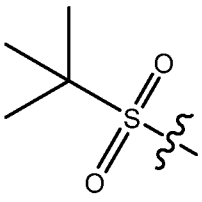
, (c) H and

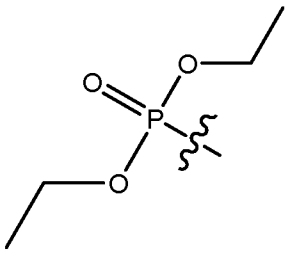


, respectively, (d) phenyl and



, respectively, (e) both





, or (f) both .

10. A composition which comprises at least one S-alkylated hepcidin peptide according to any one of claims 1 to 9.

11. A method of binding a ferroportin or inducing ferroportin internalization and degradation which comprises contacting the ferroportin with at least one S-alkylated hepcidin peptide according to any one of claims 1 to 9 or the composition according to claim 10.

12. A method of treating a disease of iron metabolism in a subject which comprises administering at least one S-alkylated hepcidin peptide according to any one of claims 1 to 9 or the composition according to claim 10 to the subject.

13. The method of claim 12, wherein the disease of iron metabolism is an iron overload disease.

14. A kit comprising at least one S-alkylated hepcidin peptide according to any one of claims 1 to 9 or the composition according to claim 10 packaged together with a reagent, a device, instructional material, or a combination thereof.

15. A complex comprising at least one S-alkylated hepcidin peptide according to any one of claims 1 to 9 bound to a ferroportin or an antibody.

16. Use of one or more S-alkylated hepcidin peptides according to any one of claims 1 to 9 or the composition according to claim 10 for the manufacture of a medicament for treating a disease of iron metabolism and/or lowering the amount of iron in a subject in need thereof.

17. One or more S-alkylated hepcidin peptides according to any one of claims 1 to 9 or the composition according to claim 10 for use in treating a disease of iron metabolism and/or lowering the amount of iron in a subject in need thereof.

18. Use of one or more S-alkylated hepcidin peptides according to any one of claims 1 to 9 or the composition according to claim 10 for the manufacture of a medicament for treating a disease of iron metabolism and/or lowering the amount of iron in a subject in need thereof, wherein the medicament is prepared to be administered at an effective daily dose as a single daily dose or as divided daily doses.

19. The use according to claim 18, wherein the effective daily dose is about 10-500 μg/kg/day and the medicament is formulated for subcutaneous injection.

20. The use according to claim 18, wherein the effective daily dose is about 10-1000 μg/kg/day and the medicament is formulated for oral, pulmonary, or mucosal administration.

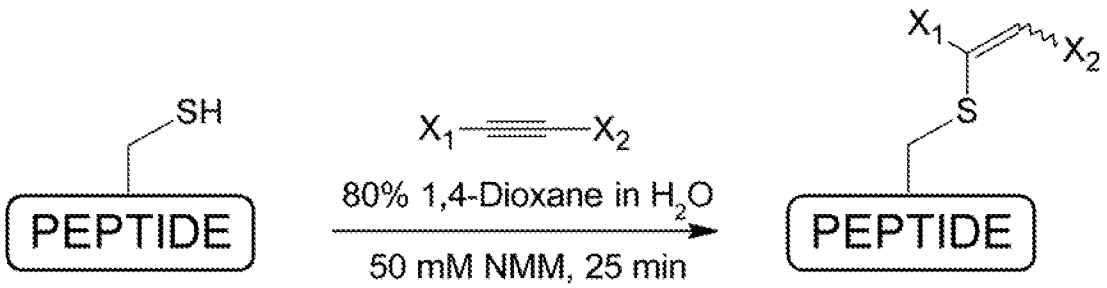

SH
PEPTIDE
X1
X2
80% 1,4-Dioxane in H2O
50 mM NMM, 25 min
X1
X2
S
PEPTIDE

Figure 1

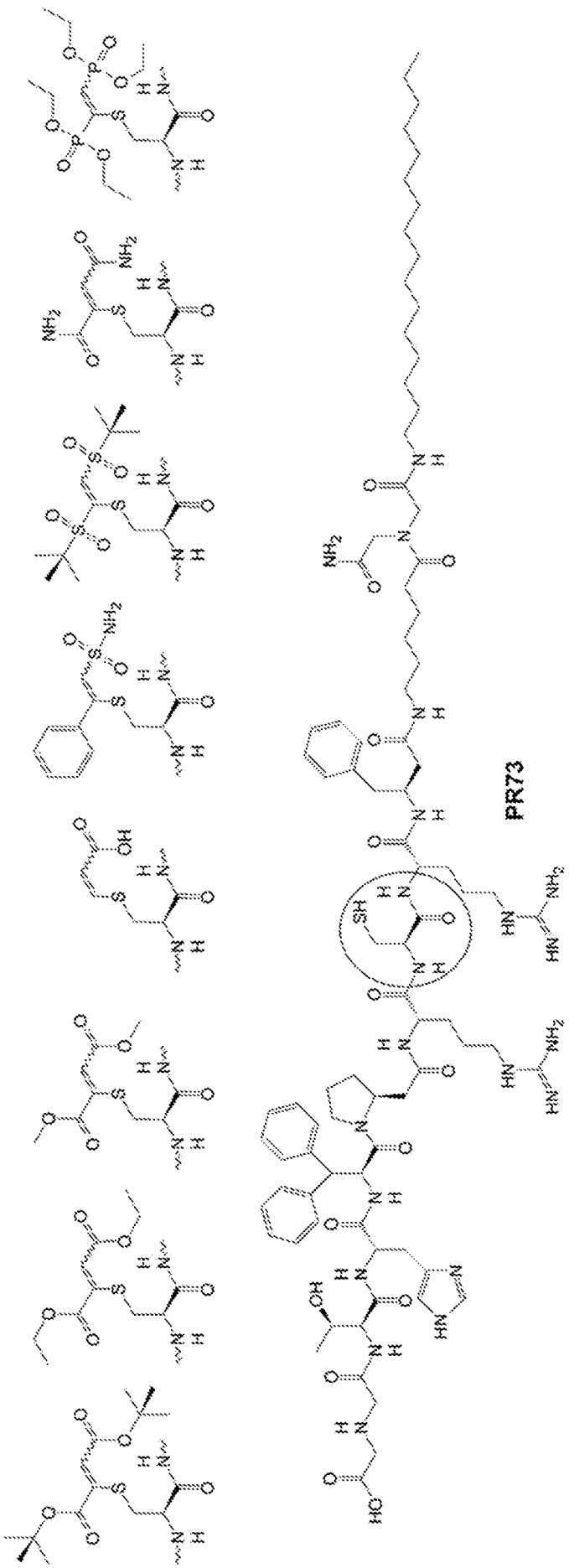

PR73SA
PR73SB
PR73SC
PR73SD
PR73SE
PR73SF
PR73SG
PR73SH
PR73

Figure 2

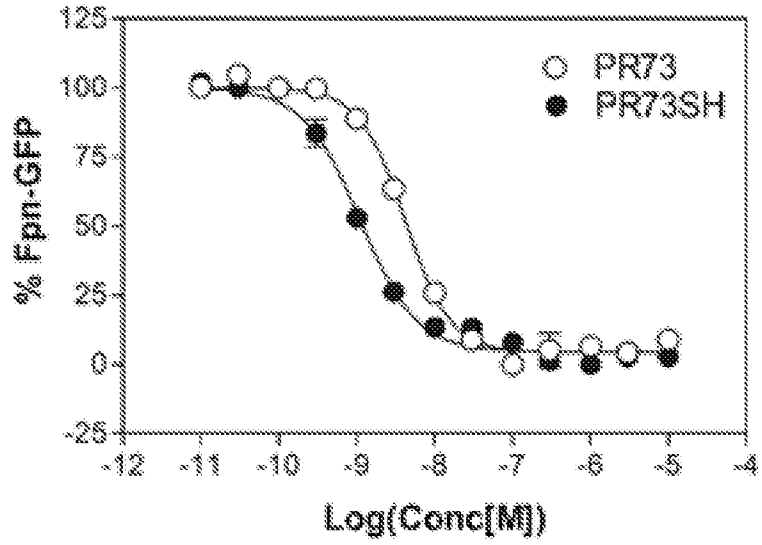


Figure 3A

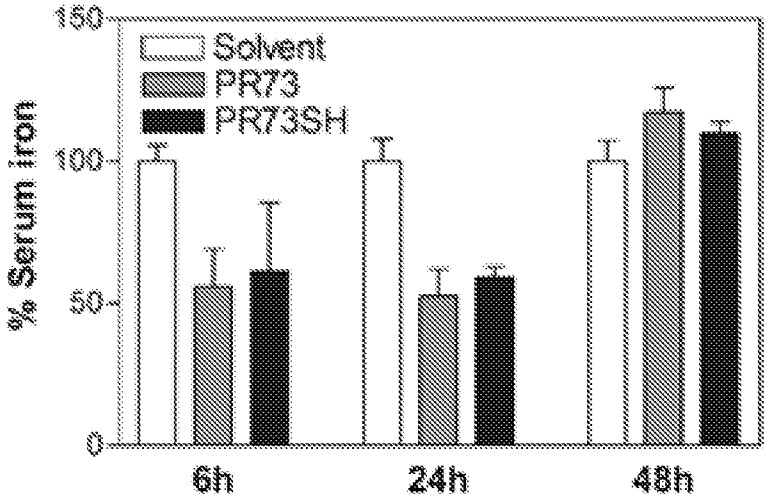


Figure 3B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/067545

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 7/06 (2016.01)
CPC - C07K 7/06 (2016.02)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 38/00, 38/08; A61P 1/16, 3/00, 3/10, 7/06, 9/04, 19/08; C07K 7/00, 7/06 (2016.01)
CPC - A61K 38/00, 38/04; C07K 7/06, 14/575 (2016.02)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/7.21; 514/5.4; 530/328, 402, 391.7 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Patbase, Google Patents, PubMed, Google.

Search terms used: hepcidin cysteine sulfur modified s-vinylcysteine s-ethenylcysteine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	FUNG et al. "Thiol-derivatized minihepcidins retain biological activity," Bioorg Med Chem Lett., 15 February 2015 (15.02.2015), Vol. 25, No. 4, Pgs. 763-766. entire document	1-3, 5, 6
A	US 2014/0336110 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA et al) 13 November 2014 (13.11.2014) entire document	1-3, 5, 6
A	THUMFORT et al. "S-Ethenyl Cysteine; An Amino Acid From Olax phyllanthi," Phytochemistry, 01 October 1993 (01.10.1993), Vol. 34, No. 3, Pgs. 657-659. entire document	1-3, 5, 6
A	PREZA et al. "Mini hepcidins are rationally designed small peptides that mimic hepcidin activity in mice and may be useful for the treatment of iron overload," The Journal of Clinical Investigation, 01 December 2011 (01.12.2011), Vol. 121, No. 12, Pgs. 4880-4889. entire document	1-3, 5, 6
A	TRIOLA et al. "Racemization-Free Synthesis of S-Alkylated Cysteines via Thiol-ene Reaction," J. Org. Chem., 01 April 2008 (01.04.2008), Vol. 73, No. 9, Pgs. 3646-3649. entire document	1-3, 5, 6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 April 2016	17 MAY 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/067545

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NO: 2 was searched

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/067545

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 10-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see Extra Sheet(s).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-3, 5, and 6 restricted to an S-alkylated hepcidin peptide of formula IA, further selected to be SEQ ID NO: 2, where the cysteine residue at position A7 is encoded by Structural Formula A, where n is 1, X1 is H, and X2 is H.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/067545

Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-9 are drawn to an S-alkylated hepcidin peptide.

The first invention of Group I+ is restricted to an S-alkylated hepcidin peptide of formula IA, further selected to be SEQ ID NO: 2, where the cysteine residue at position A7 is encoded by Structural Formula A, where n is 1, X1 is H, and X2 is H. It is believed that claims 1-3, 5, and 6 read on this first named invention and thus these claims will be searched without fee to the extent that they read on the S-alkylated hepcidin peptide of formula IA, further selected to be SEQ ID NO: 2, where the cysteine residue at position A7 is encoded by Structural Formula A, where n is 1, X1 is H, and X2 is H.

Applicant is invited to elect additional structural formulas and their respective corresponding SEQ ID NOs to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be an S-alkylated hepcidin peptide of formula IA, further selected to be SEQ ID NO: 10, where the cysteine residue at position A7 is encoded by Structural Formula A, where n is 1, X1 is H, and X2 is H. Additional structural formulas and their respective corresponding SEQ ID NOs will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element that form the bispecific tetravalent antibody, requiring the selection of alternatives where "An S-alkylated hepcidin peptide comprising or consisting of the following Structural Formula IA or IB
Al-A2-A3-A4-A5-A6-A7-A8-A9-A10 IA
A1O-A9-A8-A7-A6-A5-A4-A3-A2-A1 IB wherein Al is Asp, D-Asp, Glu, D-Glu, pyroglutamate, D-pyroglutamate, Gin, D-Gln, Asn, DAsn, or an unnatural amino acid commonly used as a substitute thereof such as bhAsp, Ida, Ida(NHPal), and N-MeAsp, preferably Ida and N-MeAsp; A2 is Thr, D-Thr, Ser, D-Ser, Val, D-Val, Ile, D-Ile, Ala, D-Ala or an unnatural amino acid commonly used as a substitute thereof such as Tle, Inp, Chg, bhThr, and NMe Thr; A3 is His, D-His, Asn, D-Asn, Arg, D-Arg, or an unnatural amino acid commonly used as a substitute thereof such as L-His(n-Me), D-His(7r-Me), L-His(t-Me), or DHis (t-Me); A4 is Phe, D-Phe, Leu, D-Leu, Ile, D-Ile, Trp, D-Trp, Tyr, D-Tyr, or an unnatural amino acid commonly used as a substitute thereof such as Phg, bhPhe, Dpa, Bip, INal, 2Nal, bhDpa, Amc, PheF5, hPhe, Igi, or cyclohexylalanine, preferably Dpa; A5 is Pro, D-Pro, Ser, D-Ser, or an unnatural amino acid commonly used as a substitute thereof such as Oic, bhPro, trans-4-PhPro, cis-4-PhPro, cis-5-PhPro, and Idc, preferably bhPro; A6 is Arg, D-Arg, Ile, D-Ile, Leu, D-Leu, Thr, D-Thr, Lys, D-Lys, Val, D-Val, or an unnatural amino acid commonly used as a substitute thereof such as D-NO,odimethyl-arginine, L-No,mo-dimethyl-arginine, D-homoarginine, L-homoarginine, D-norarginine, L-norarginine, citrulline, a modified Arg wherein the guanidinium group is modified or substituted, Norleucine, norvaline, bhIle, Ach, N-MeArg, and N-MeIle, preferably Arg; A7 is Cys, D-Cys, Ser, D-Ser, Ala, D-Ala, or an unnatural amino acid commonly used as a substitute thereof such as Cys (S-tBut), homoCys, Pen, (D)Pen, preferably Stertiary butyl-cysteine, Cys(S-S-Pal), Cys(S-S-cysteamine-Pal), Cys(S-S-Cys- NHPal), and Cys(S-S-Cys); A8 is Arg, D-Arg, Ile, D-Ile, Leu, D-Leu, Thr, D-Thr, Lys, D-Lys, Val, D-Val, or an unnatural amino acid commonly used as a substitute thereof such as D-Nzo,odimethyl-arginine, L-No,mo-dimethyl-arginine, D-homoarginine, L-homoarginine, norarginine, L-norarginine, citrulline, a modified Arg wherein the guanidinium group is modified or substituted, Norleucine, norvaline, bhIle, Ach, N-MeArg, and N-MeIle, preferably Arg; A9 is Phe, D-Phe, Leu, D-Leu, Ile, D-Ile, Tyr, D-Tyr, Trp, D-Trp, Phe-Ra, D-Phe-Ra, Dpa-Ra, D-Dpa-Ra, Trp-Ra, bhPhe-Ra, or an unnatural amino acid commonly used as a substitute thereof such as PheF5, N-MePhe, benzylamide, 2-aminoindane, bhPhe, Dpa, Bip, INal, 2Nal, bhDpa, and cyclohexylalanine, which may or may not have Ra linked thereto, preferably bhPhe and bhPhe-Ra, wherein Ra is palmitoyl-PEG-, wherein PEG is PEG 11 or miniPEG3, palmitoyl-PEG-PEG, wherein PEG is PEG 11 or miniPEG3, butanoyl (C4)-PEGJ 1-, octanoyl (C8, Caprylic)-PEGI 1-, palmitoyl (C16)-PEGI 1-, or tetracosanoyl (C24, Lignoceric)-PEG11-; and ATO is Cys, D-Cys, Ser, D-Ser, Ala, D-Ala, or an unnatural amino acid such as Ida, Ida(NTPal)Ahx, and Ida (NBzl2)Ahx; and at least one of the amino acid residues Al to A10 has Structural Formula A: (See structure of claim 1), wherein n is 1 or 2 and one or more of the hydrogens bonded to the Cn atom(s) may be substituted with a (Cl-C3)alkyl, Xi and X2 are each independently selected from the group consisting of H, alkyl, alkoxy, alkoxycarbonyl, cycloalkyl, aryl, heteroaryl, heterocycloalkyl, acyl, sulfonyl, alkylsulfonyl, alkylamino, alkylaminocarbonyl, dialkylaninocarbonyl, carboxyl, and carbamoyl;
wherein the carboxy-terminal amino acid is in amide or carboxy- form; and wherein Al, Al to A2, A10, or a combination thereof are optionally absent."

The Groups I+ share the technical features of an S-alkylated hepcidin peptide comprising or consisting of the following Structural Formula IA or IB Al-A2-A3-A4-A5-A6-A7-A8-A9-A10 IA, A1O-A9-A8-A7-A6-A5-A4-A3-A2-A1 IB. However, these shared technical features do not represent a contribution over the prior art.

Specifically, US 2014/0336110 A1 to The Regents of the University of California et al., discloses an S-alkylated hepcidin peptide (Hep9C7-tBut (t-butyl-blocked cysteine) or with a cysteine modified by disulfide coupled tertiary butyl, Para. [0070]; Disclosed herein are peptides which exhibit hepcidin activity, Abstract) comprising or consisting of the following Structural Formula IA or IB A1-A2-A3-A4-A5-A6-A7-A8-A9-A10 IA, A10-A9-A8-A7-A6-A5-A4-A3-A2-A1 IB (The present invention provides peptides, which may be isolated and/ or purified, comprising, consisting essentially or consisting of the following Structural Formula IA or IB, Para. [0012]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.