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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 October 2022 (27.10.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/223969 A1

(51) International Patent Classification:
C07K 7/08 (2006.01) *A61P 35/00* (2006.01)
A61K 47/64 (2017.01)

(21) International Application Number:
PCT/GB2022/050995

(22) International Filing Date:
20 April 2022 (20.04.2022)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/177,185 20 April 2021 (20.04.2021) US

(71) Applicant: **BICYCLETX LIMITED** [GB/GB]; Building 900, Babraham Research Campus, Cambridge CB22 3AT (GB).

(72) Inventors: **MCDONNELL, Kevin**; c/o BicycleTx Limited, Building 900, Babraham Research Campus, Cambridge CB22 3AT (GB). **MUDD, Gemma**; c/o BicycleTx Limited, Building 900, Babraham Research Campus, Cambridge CB22 3AT (GB). **SKYNNER, Michael**; c/o BicycleTx Limited, Building 900, Babraham Research Campus, Cambridge CB22 3AT (GB). **WATCHAM, Sophie**; c/o BicycleTx Limited, Building 900, Babraham Research Campus, Cambridge CB22 3AT (GB).

(74) Agent: **GIBSON, Mark** et al.; Sagittarius IP, Marlow International, Parkway, Marlow Buckinghamshire SL7 1YL (GB).

(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, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, 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, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, 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,

(54) Title: BICYCLIC PEPTIDE LIGANDS SPECIFIC FOR P-SELECTIN

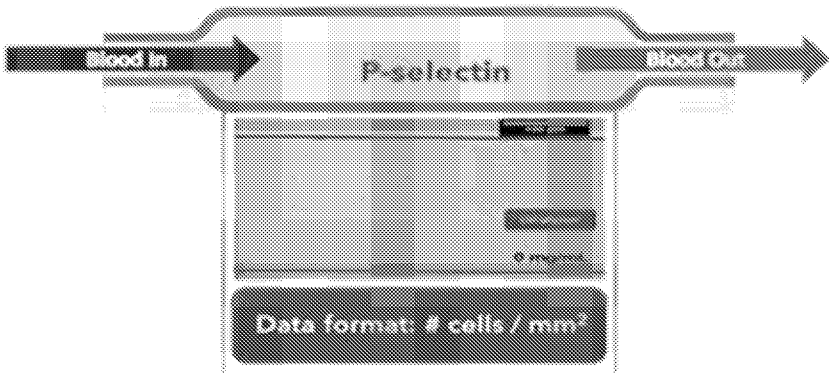


FIGURE 1

(57) Abstract: The present invention relates to polypeptides which are covalently bound to molecular scaffolds such that two or more peptide loops are subtended between attachment points to the scaffold. In particular, the invention describes peptides which bind to P-selectin. The invention also relates to multimeric binding complexes of polypeptides which are covalently bound to molecular scaffolds such that two or more peptide loops are subtended between attachment points to the scaffold that are binders of P-selectin. The invention also includes drug conjugates comprising said peptides and complexes, conjugated to one or more effector and/or functional groups, to pharmaceutical compositions comprising said peptide ligands, complexes and drug conjugates and the use of said peptide ligands and drug conjugates in preventing, suppressing or treating a disease or disorder mediated by a cell adhesion molecule, such as P-selectin, including vaso-occlusive crisis and sickle cell disease-related conditions, cancer, or COVID-19.

WO 2022/223969 A1

[Continued on next page]

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).

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))*

BICYCLIC PEPTIDE LIGANDS SPECIFIC FOR P-SELECTIN

FIELD OF THE INVENTION

The present invention relates to polypeptides which are covalently bound to molecular scaffolds such that two or more peptide loops are subtended between attachment points to the scaffold. In particular, the invention describes peptides which bind to P-selectin. The invention also relates to multimeric binding complexes of polypeptides which are covalently bound to molecular scaffolds such that two or more peptide loops are subtended between attachment points to the scaffold that are binders of P-selectin. The invention also includes drug conjugates comprising said peptides and complexes, conjugated to one or more effector and/or functional groups, to pharmaceutical compositions comprising said peptide ligands, complexes and drug conjugates and the use of said peptide ligands and drug conjugates in preventing, suppressing or treating a disease or disorder mediated by a cell adhesion molecule, such as P-selectin, including vaso-occlusive crisis and sickle cell disease-related conditions, cancer, or COVID-19.

BACKGROUND OF THE INVENTION

Cyclic peptides are able to bind with high affinity and specificity to protein targets and hence are an attractive molecule class for the development of therapeutics. In fact, several cyclic peptides are already successfully used in the clinic, as for example the antibacterial peptide vancomycin, the immunosuppressant drug cyclosporine or the anti-cancer drug octreotide (Driggers *et al.* (2008), Nat. Rev. Drug. Discov. *7*(7), 608-24). Good binding properties result from a relatively large interaction surface formed between the peptide and the target as well as the reduced conformational flexibility of the cyclic structures. Typically, macrocycles bind to surfaces of several hundred square angstrom, as for example the cyclic peptide CXCR4 antagonist CVX15 (400 $Å^2$; Wu *et al.* (2007), Science 330, 1066-71), a cyclic peptide with the Arg-Gly-Asp motif binding to integrin αVb3 (355 $Å^2$) (Xiong *et al.* (2002), Science *296*(5565), 151-5) or the cyclic peptide inhibitor upain-1 binding to urokinase-type plasminogen activator (603 $Å^2$; Zhao *et al.* (2007), J. Struct. Biol. 160(1), 1-10).

Due to their cyclic configuration, peptide macrocycles are less flexible than linear peptides, leading to a smaller loss of entropy upon binding to targets and resulting in a higher binding affinity. The reduced flexibility also leads to locking target-specific conformations, increasing binding specificity compared to linear peptides. This effect has been exemplified by a potent and selective inhibitor of matrix metalloproteinase 8 (MMP-8) which lost its selectivity over other MMPs when its ring was opened (Cherney *et al.* (1998), J. Med. Chem. 41(11), 1749-51). The favourable binding properties achieved through macrocyclization are even more

pronounced in multicyclic peptides having more than one peptide ring as for example in vancomycin, nisin and actinomycin.

Different research teams have previously tethered polypeptides with cysteine residues to a synthetic molecular structure (Kemp and McNamara (1985), J. Org. Chem; Timmerman *et al.* (2005), ChemBioChem). Meloen and co-workers had used tris(bromomethyl)benzene and related molecules for rapid and quantitative cyclisation of multiple peptide loops onto synthetic scaffolds for structural mimicry of protein surfaces (Timmerman *et al.* (2005), ChemBioChem). Methods for the generation of candidate drug compounds wherein said compounds are generated by linking cysteine containing polypeptides to a molecular scaffold as for example 1,1',1''-(1,3,5-triazinane-1,3,5-triyl)triprop-2-en-1-one (TATA) (Heinis *et al.*(2014) Angewandte Chemie, International Edition 53(6) 1602-1606).

Phage display-based combinatorial approaches have been developed to generate and screen large libraries of bicyclic peptides to targets of interest (Heinis *et al.* (2009), Nat. Chem. Biol. 5(7), 502-7 and WO 2009/098450). Briefly, combinatorial libraries of linear peptides containing three cysteine residues and two regions of six random amino acids (Cys-$(Xaa)_6$-Cys-$(Xaa)_6$-Cys) were displayed on phage and cyclised by covalently linking the cysteine side chains to a small molecule scaffold.

SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a peptide ligand specific for P-selectin comprising a polypeptide comprising at least three reactive groups, separated by at least two loop sequences, and a molecular scaffold which forms covalent bonds with the reactive groups of the polypeptide such that at least two polypeptide loops are formed on the molecular scaffold.

According to a further aspect of the invention, there is provided a multimeric binding complex which comprises at least two peptide ligands, wherein at least one peptide ligand is specific for P-selectin as defined herein and said peptide ligands may be the same or different, each of which comprises a polypeptide comprising at least three reactive groups, separated by at least two loop sequences, and a molecular scaffold which forms covalent bonds with the reactive groups of the polypeptide such that at least two polypeptide loops are formed on the molecular scaffold.

According to a yet further aspect of the invention, there is provided a drug conjugate comprising the peptide ligand or multimeric binding complex as defined herein, conjugated to one or more effector and/or functional groups.

According to a yet further aspect of the invention, there is provided a pharmaceutical composition comprising a peptide ligand, multimeric binding complex or drug conjugate as defined herein in combination with one or more pharmaceutically acceptable excipients.

According to a further aspect of the invention, there is provided a peptide ligand, multimeric binding complex, drug conjugate or pharmaceutical composition as defined herein for use in preventing, suppressing or treating a disease or disorder mediated by P-selectin.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Schematic representation of P-selectin flow adhesion assay.

Figure 2: Effect of BCY12262 on isolated white blood cell flow adhesion on P-selectin. Fold change of AI (adhesion index: cells/mm²) from baseline (no-drug control, not displayed on graph). Mean of 3 data points displayed with error bars denoting the standard deviation. Dashed lines represent the mean of the samples displayed.

DETAILED DESCRIPTION OF THE INVENTION

Peptide Ligands

According to a first aspect of the invention, there is provided a peptide ligand specific for P-selectin comprising a polypeptide comprising at least three reactive groups, separated by at least two loop sequences, and a molecular scaffold which forms covalent bonds with the reactive groups of the polypeptide such that at least two polypeptide loops are formed on the molecular scaffold.

In one embodiment, said reactive groups comprise cysteine residues.

In a further embodiment, said peptide ligand comprises the motif WCDV. The WCDV amino acid motif has previously been shown to confer binding of linear peptide ligands to P-selectin. However, the present inventors surprisingly found that the binding affinity of such a motif, and modified derivatives thereof is enhanced when incorporated within the bicyclic peptides as described herein. In a yet further embodiment, said peptide ligand comprises a modified derivative of the motif WCDV.

In a further embodiment, said loop sequences comprise 4 or 6 amino acids.

In one embodiment, said loop sequences comprise three cysteine residues separated by two loop sequences the first of which consists of 4 amino acids and the second of which consists of 6 amino acids.

In one embodiment, the peptide ligand comprises an amino acid sequence of:

C_i-X_1-X_2-X_3-X_4-C_{ii}-D-V-X_5-X_6-X_7-X_8-C_{iii} (SEQ ID NO: 1),

wherein

X_1 represents D or Y;

X_2 represents A or M;

X_3 represents D or E;

X_4 represents W, 1Nal or Trp(Me);

X_5 represents P or T;

X_6 represents S or D;

X_7 represents L or Y;

X_8 represents P or G;

wherein 1Nal represents 1-naphthylalanine, Trp(Me) represents methyl-tryptophan and C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, or a modified derivative, or a pharmaceutically acceptable salt thereof.

In a further embodiment, X_4 represents W.

In a yet further embodiment, the peptide ligand of C_i-X_1-X_2-X_3-X_4-C_{ii}-D-V-X_5-X_6-X_7-X_8-C_{iii} (SEQ ID NO: 1) comprises an amino acid sequence selected from:

C_iDAD[1Nal]C_{ii}DVPSLPC_{iii} (SEQ ID NO: 2);

C_iDADWC_{ii}DVPSLPC_{iii} (SEQ ID NO: 3);

C_iYME[1Nal]C_{ii}DVTDYGC_{iii} (SEQ ID NO: 4);

C_iYME[Trp(Me)]C_{ii}DVTDYGC_{iii} (SEQ ID NO: 5); and

C_iYMEWC_{ii}DVTDYGC_{iii} (SEQ ID NO: 6);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, or a modified derivative, or a pharmaceutically acceptable salt thereof).

In a further embodiment, the peptide ligand comprises N- and/or C-terminal additions and is selected from:

A-(SEQ ID NO: 2)-A (herein referred to as BCY12027);

H_2N-A-(SEQ ID NO: 2)-A-[K(PYA)] (herein referred to as BCY12026);

A-(SEQ ID NO: 3)-A (herein referred to as BCY11648);

H_2N-A-(SEQ ID NO: 3)-A-[K(PYA)] (herein referred to as BCY12025);
Ac-A-(SEQ ID NO: 4)-A (herein referred to as BCY11279);
A-(SEQ ID NO: 4)-A-[K(PYA)] (herein referred to as BCY11890);
Ac-A-(SEQ ID NO: 5)-A (herein referred to as BCY11281);
Ac-(SEQ ID NO: 6) (herein referred to as BCY9717);
A-(SEQ ID NO: 6)-A (herein referred to as BCY10194);
A-(SEQ ID NO: 6)-A-[K(PYA)] (herein referred to as BCY18041);
[PYA]-A-(SEQ ID NO: 6)-A-NH_2 (herein referred to as BCY10910); and
Ac-A-(SEQ ID NO: 6)-[K(PYA)]-NH_2 (herein referred to as BCY10911),

wherein PYA represents 4-pentynoic acid.

In a yet further embodiment, the peptide ligand comprises N- and/or C-terminal additions and is selected from:

A-(SEQ ID NO: 2)-A (herein referred to as BCY12027);
H_2N-A-(SEQ ID NO: 2)-A-[K(PYA)] (herein referred to as BCY12026);
A-(SEQ ID NO: 3)-A (herein referred to as BCY11648);
H_2N-A-(SEQ ID NO: 3)-A-[K(PYA)] (herein referred to as BCY12025);
Ac-A-(SEQ ID NO: 4)-A (herein referred to as BCY11279);
Ac-A-(SEQ ID NO: 5)-A (herein referred to as BCY11281);
Ac-(SEQ ID NO: 6) (herein referred to as BCY9717);
A-(SEQ ID NO: 6)-A (herein referred to as BCY10194);
[PYA]-A-(SEQ ID NO: 6)-A-NH_2 (herein referred to as BCY10910); and
Ac-A-(SEQ ID NO: 6)-[K(PYA)]-NH_2 (herein referred to as BCY10911),

wherein PYA represents 4-pentynoic acid.

The peptides of this embodiment display good binding to P-selectin (see Table 6).

In a particular embodiment, the peptide ligand is selected from: BCY11648, BCY12027, BCY12025 and BCY12026. In a further embodiment, the peptide ligand is selected from: BCY12027 and BCY12026. The peptides of these embodiments display very good binding to P-selectin, for example BCY12027 displays a high affinity for P-selectin with a K_D of less than 10nM (see Table 6).

In a further embodiment, the pharmaceutically acceptable salt is selected from the free acid or the sodium, potassium, calcium or ammonium salt.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art, such as in the arts of peptide chemistry, cell culture and phage display, nucleic acid chemistry and biochemistry. Standard techniques are used for molecular biology, genetic and biochemical methods (see Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 3rd ed., 2001, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th ed., John Wiley & Sons, Inc.), which are incorporated herein by reference.

Numbering

When referring to amino acid residue positions within the peptides of the invention, cysteine residues (C_i, C_{ii} and C_{iii}) are omitted from the numbering as they are invariant, therefore, the numbering of amino acid residues within the peptides of the invention is referred to as below:

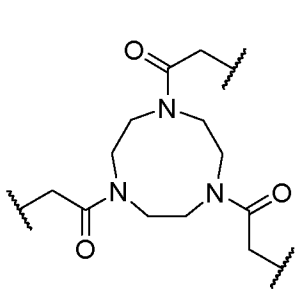
$-C_i-D_1-A_2-D_3-1Nal_4-C_{ii}-D_5-V_6-P_7-S_8-L_9-P_{10}-C_{iii}-$ (SEQ ID NO: 2).

Molecular Format

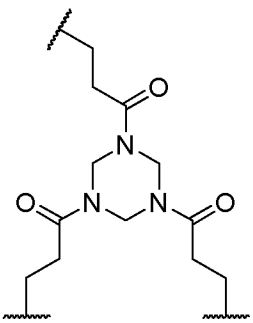
N- or C-terminal extensions to the bicycle core sequence are added to the left or right side of the sequence, separated by a hyphen. For example, an N-terminal biotin-G-Sar_5 tail would be denoted as:

[Biot]-G-[Sar_5]-A-(SEQ ID NO: X).

For the purpose of this description, all bicyclic peptides are assumed to be cyclised with either 1,1',1''-(1,3,5-triazinane-1,3,5-triyl)triprop-2-en-1-one (TATA) or 1,1',1''-(1,4,7-triazonane-1,4,7-triyl)tris(2-chloroethan-1-one) (TCAZ) or the bromo derivative 1,1',1''-(1,4,7-triazonane-1,4,7-triyl)tris(2-bromothan-1-one) (TBAZ):



TCAZ/TBAZ



TATA

and yielding a tri-substituted structure. However, as will be clear from the descriptions of the invention presented herein, cyclisation may be performed with any suitable molecular scaffold which forms covalent bonds with the reactive groups of the polypeptide such that at least two polypeptide loops are formed. Cyclisation occurs on C_i, C_{ii}, and C_{iii}.

In one embodiment, the molecular scaffold is TATA and the bicyclic peptide is selected from: BCY11279, BCY11281, BCY9717, BCY10194, BCY10910 and BCY10911.

In an alternative embodiment, the molecular scaffold is TCAZ and the bicyclic peptide is selected from: BCY12027, BCY11648, BCY12025 and BCY12026.

Inversed Peptide Sequences

In light of the disclosure in Nair *et al.* (2003) J. Immunol. 170(3), 1362-1373, it is envisaged that the peptide sequences disclosed herein would also find utility in their retro-inverso form. For example, the sequence is reversed (i.e. N-terminus become C-terminus and *vice versa*) and their stereochemistry is likewise also reversed (i.e. D-amino acids become L-amino acids and *vice versa*).

Peptide Ligand Definition

A peptide ligand, as referred to herein, refers to a peptide, peptidic or peptidomimetic covalently bound to a molecular scaffold. Typically, such peptides, peptidics or peptidomimetics comprise a peptide having natural or non-natural amino acids, two or more reactive groups (i.e. cysteine residues) which are capable of forming covalent bonds to the scaffold, and a sequence subtended between said reactive groups which is referred to as the loop sequence, since it forms a loop when the peptide, peptidic or peptidomimetic is bound to the scaffold. In the present case, the peptides, peptidics or peptidomimetics comprise at least three cysteine residues (referred to herein as C_i, C_{ii} and C_{iii}), and form at least two loops on the scaffold.

Advantages of the Peptide Ligands

Certain bicyclic peptides of the present invention have a number of advantageous properties which enable them to be considered as suitable drug-like molecules for injection, inhalation, nasal, ocular, oral or topical administration. Such advantageous properties include:

- Species cross-reactivity. This is a typical requirement for preclinical pharmacodynamics and pharmacokinetic evaluation;
- Protease stability. Bicyclic peptide ligands should in most circumstances demonstrate stability to plasma proteases, epithelial ("membrane-anchored") proteases, gastric and intestinal proteases, lung surface proteases, intracellular proteases and the like. Protease stability should be maintained between different species such that a bicyclic peptide lead candidate can be developed in animal models as well as administered with confidence to humans;

- Desirable solubility profile. This is a function of the proportion of charged and hydrophilic versus hydrophobic residues and intra/inter-molecular H-bonding, which is important for formulation and absorption purposes; and
- An optimal plasma half-life in the circulation. Depending upon the clinical indication and treatment regimen, it may be required to develop a bicyclic peptide with short or prolonged *in vivo* exposure times for the management of either chronic or acute disease states. The optimal exposure time will be governed by the requirement for sustained exposure (for maximal therapeutic efficiency) versus the requirement for short exposure times to minimise toxicological effects arising from sustained exposure to the agent.

Pharmaceutically Acceptable Salts

It will be appreciated that salt forms are within the scope of this invention, and references to peptide ligands include the salt forms of said ligands.

The salts of the present invention can be synthesized from the parent compound that contains a basic or acidic moiety by conventional chemical methods such as methods described in *Pharmaceutical Salts: Properties, Selection, and Use*, P. Heinrich Stahl (Editor), Camille G. Wermuth (Editor), ISBN: 3-90639-026-8, Hardcover, 388 pages, August 2002. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with the appropriate base or acid in water or in an organic solvent, or in a mixture of the two.

Acid addition salts (mono- or di-salts) may be formed with a wide variety of acids, both inorganic and organic. Examples of acid addition salts include mono- or di-salts formed with an acid selected from the group consisting of acetic, 2,2-dichloroacetic, adipic, alginic, ascorbic (e.g. L-ascorbic), L-aspartic, benzenesulfonic, benzoic, 4-acetamidobenzoic, butanoic, (+) camphoric, camphor-sulfonic, (+)-(1*S*)-camphor-10-sulfonic, capric, caproic, caprylic, cinnamic, citric, cyclamic, dodecylsulfuric, ethane-1,2-disulfonic, ethanesulfonic, 2-hydroxyethanesulfonic, formic, fumaric, galactaric, gentisic, glucoheptonic, D-gluconic, glucuronic (e.g. D-glucuronic), glutamic (e.g. L-glutamic), α-oxoglutaric, glycolic, hippuric, hydrohalic acids (e.g. hydrobromic, hydrochloric, hydriodic), isethionic, lactic (e.g. (+)-L-lactic, (±)-DL-lactic), lactobionic, maleic, malic, (-)-L-malic, malonic, (±)-DL-mandelic, methanesulfonic, naphthalene-2-sulfonic, naphthalene-1,5-disulfonic, 1-hydroxy-2-naphthoic, nicotinic, nitric, oleic, orotic, oxalic, palmitic, pamoic, phosphoric, propionic, pyruvic, L-pyroglutamic, salicylic, 4-amino-salicylic, sebacic, stearic, succinic, sulfuric, tannic, (+)-L-tartaric, thiocyanic, *p*-toluenesulfonic, undecylenic and valeric acids, as well as acylated amino acids and cation exchange resins.

One particular group of salts consists of salts formed from acetic, hydrochloric, hydriodic, phosphoric, nitric, sulfuric, citric, lactic, succinic, maleic, malic, isethionic, fumaric, benzenesulfonic, toluenesulfonic, sulfuric, methanesulfonic (mesylate), ethanesulfonic, naphthalenesulfonic, valeric, propanoic, butanoic, malonic, glucuronic and lactobionic acids. One particular salt is the hydrochloride salt. Another particular salt is the acetate salt.

If the compound is anionic, or has a functional group which may be anionic (e.g. -COOH may be $-COO^-$), then a salt may be formed with an organic or inorganic base, generating a suitable cation. Examples of suitable inorganic cations include, but are not limited to, alkali metal ions such as Li^+, Na^+ and K^+, alkaline earth metal cations such as Ca^{2+} and Mg^{2+}, and other cations such as Al^{3+} or Zn^+. Examples of suitable organic cations include, but are not limited to, ammonium ion (i.e. NH_4^+) and substituted ammonium ions (e.g. NH_3R^+, $NH_2R_2^+$, NHR_3^+, NR_4^+). Examples of some suitable substituted ammonium ions are those derived from: methylamine, ethylamine, diethylamine, propylamine, dicyclohexylamine, triethylamine, butylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine, benzylamine, phenylbenzylamine, choline, meglumine, and tromethamine, as well as amino acids, such as lysine and arginine. An example of a common quaternary ammonium ion is $N(CH_3)_4^+$.

Where the peptides of the invention contain an amine function, these may form quaternary ammonium salts, for example by reaction with an alkylating agent according to methods well known to the skilled person. Such quaternary ammonium compounds are within the scope of the peptides of the invention.

Modified Derivatives

It will be appreciated that modified derivatives of the peptide ligands as defined herein are within the scope of the present invention. Examples of such suitable modified derivatives include one or more modifications selected from: N-terminal and/or C-terminal modifications; replacement of one or more amino acid residues with one or more non-natural amino acid residues (such as replacement of one or more polar amino acid residues with one or more isosteric or isoelectronic amino acids; replacement of one or more non-polar amino acid residues with other non-natural isosteric or isoelectronic amino acids); addition of a spacer group; replacement of one or more oxidation sensitive amino acid residues with one or more oxidation resistant amino acid residues; replacement of one or more amino acid residues with one or more replacement amino acids, such as an alanine, replacement of one or more L-amino acid residues with one or more D-amino acid residues; N-alkylation of one or more amide bonds within the bicyclic peptide ligand; replacement of one or more peptide bonds with a surrogate bond; peptide backbone length modification; substitution of the hydrogen on the

alpha-carbon of one or more amino acid residues with another chemical group; modification of amino acids such as cysteine, lysine, glutamate/aspartate and tyrosine with suitable amine, thiol, carboxylic acid and phenol-reactive reagents so as to functionalise said amino acids; and introduction or replacement of amino acids that introduce orthogonal reactivities that are suitable for functionalisation, for example azide or alkyne-group bearing amino acids that allow functionalisation with alkyne or azide-bearing moieties, respectively.

In one embodiment, the modified derivative comprises an N-terminal and/or C-terminal modification. In a further embodiment, wherein the modified derivative comprises an N-terminal modification using suitable amino-reactive chemistry, and/or C-terminal modification using suitable carboxy-reactive chemistry. In a further embodiment, said N-terminal or C-terminal modification comprises addition of an effector group, including but not limited to a cytotoxic agent, a radiochelator or a chromophore.

In a further embodiment, the modified derivative comprises an N-terminal modification. In a further embodiment, the N-terminal modification comprises an N-terminal acetyl group. In this embodiment, the N-terminal residue is capped with acetic anhydride or other appropriate reagents during peptide synthesis leading to a molecule which is N-terminally acetylated. This embodiment provides the advantage of removing a potential recognition point for aminopeptidases and avoids the potential for degradation of the bicyclic peptide.

In an alternative embodiment, the N-terminal modification comprises the addition of a molecular spacer group which facilitates the conjugation of effector groups and retention of potency of the bicyclic peptide to its target.

In a further embodiment, the modified derivative comprises a C-terminal modification. In a further embodiment, the C-terminal modification comprises an amide group. In this embodiment, the C-terminal residue is synthesized as an amide during peptide synthesis leading to a molecule which is C-terminally amidated. This embodiment provides the advantage of removing a potential recognition point for carboxypeptidase and reduces the potential for proteolytic degradation of the bicyclic peptide.

In one embodiment, the modified derivative comprises replacement of one or more amino acid residues with one or more non-natural amino acid residues. In this embodiment, non-natural amino acids may be selected having isosteric/isoelectronic side chains which are neither recognised by degradative proteases nor have any adverse effect upon target potency.

Alternatively, non-natural amino acids may be used having constrained amino acid side chains, such that proteolytic hydrolysis of the nearby peptide bond is conformationally and sterically impeded. In particular, these concern proline analogues, bulky sidechains, Cα-disubstituted derivatives (for example, aminoisobutyric acid, Aib), and cyclo amino acids, a simple derivative being amino-cyclopropylcarboxylic acid.

In one embodiment, the modified derivative comprises the addition of a spacer group. In a further embodiment, the modified derivative comprises the addition of a spacer group to the N-terminal cysteine (C_i) and/or the C-terminal cysteine (C_{iii}).

In one embodiment, the modified derivative comprises replacement of one or more oxidation sensitive amino acid residues with one or more oxidation resistant amino acid residues. In a further embodiment, the modified derivative comprises replacement of a tryptophan residue with a naphthylalanine or alanine residue. This embodiment provides the advantage of improving the pharmaceutical stability profile of the resultant bicyclic peptide ligand.

In one embodiment, the modified derivative comprises replacement of one or more charged amino acid residues with one or more hydrophobic amino acid residues. In an alternative embodiment, the modified derivative comprises replacement of one or more hydrophobic amino acid residues with one or more charged amino acid residues. The correct balance of charged versus hydrophobic amino acid residues is an important characteristic of the bicyclic peptide ligands. For example, hydrophobic amino acid residues influence the degree of plasma protein binding and thus the concentration of the free available fraction in plasma, while charged amino acid residues (in particular arginine) may influence the interaction of the peptide with the phospholipid membranes on cell surfaces. The two in combination may influence half-life, volume of distribution and exposure of the peptide drug, and can be tailored according to the clinical endpoint. In addition, the correct combination and number of charged versus hydrophobic amino acid residues may reduce irritation at the injection site (if the peptide drug has been administered subcutaneously).

In one embodiment, the modified derivative comprises replacement of one or more L-amino acid residues with one or more D-amino acid residues. This embodiment is believed to increase proteolytic stability by steric hindrance and by a propensity of D-amino acids to stabilise β-turn conformations (Tugyi *et al.* (2005) PNAS, 102(2), 413–418).

In one embodiment, the modified derivative comprises removal of any amino acid residues and substitution with alanines, such as D-alanines. This embodiment provides the advantage

of identifying key binding residues and removing potential proteolytic attack site(s).

It should be noted that each of the above mentioned modifications serve to deliberately improve the potency or stability of the peptide. Further potency improvements based on modifications may be achieved through the following mechanisms:

- Incorporating hydrophobic moieties that exploit the hydrophobic effect and lead to lower off rates, such that higher affinities are achieved;
- Incorporating charged groups that exploit long-range ionic interactions, leading to faster on rates and to higher affinities (see for example Schreiber *et al.*, *Rapid, electrostatically assisted association of proteins* (1996), Nature Struct. Biol. 3, 427-31); and
- Incorporating additional constraint into the peptide, by for example constraining side chains of amino acids correctly such that loss in entropy is minimal upon target binding, constraining the torsional angles of the backbone such that loss in entropy is minimal upon target binding and introducing additional cyclisations in the molecule for identical reasons.

(for reviews see Gentilucci *et al.*, Curr. Pharmaceutical Design, (2010), 16, 3185-203, and Nestor *et al.*, Curr. Medicinal Chem (2009), 16, 4399-418).

Isotopic Variations

The present invention includes all pharmaceutically acceptable (radio)isotope-labelled peptide ligands of the invention, wherein one or more atoms are replaced by atoms having the same atomic number, but an atomic mass or mass number different from the atomic mass or mass number usually found in nature, and peptide ligands of the invention, wherein metal chelating groups are attached (termed “effector”) that are capable of holding relevant (radio)isotopes, and peptide ligands of the invention, wherein certain functional groups are covalently replaced with relevant (radio)isotopes or isotopically labelled functional groups.

Examples of isotopes suitable for inclusion in the peptide ligands of the invention comprise isotopes of hydrogen, such as ^{2}H (D) and ^{3}H (T), carbon, such as ^{11}C, ^{13}C and ^{14}C, chlorine, such as ^{36}Cl, fluorine, such as ^{18}F, iodine, such as ^{123}I, ^{125}I and ^{131}I, nitrogen, such as ^{13}N and ^{15}N, oxygen, such as ^{15}O, ^{17}O and ^{18}O, phosphorus, such as ^{32}P, sulphur, such as ^{35}S, copper, such as ^{64}Cu, gallium, such as ^{67}Ga or ^{68}Ga, yttrium, such as ^{90}Y and lutetium, such as ^{177}Lu, and Bismuth, such as ^{213}Bi.

Certain isotopically-labelled peptide ligands of the invention, for example, those incorporating a radioactive isotope, are useful in drug and/or substrate tissue distribution studies, and to clinically assess the presence and/or absence of the DLL3 target on diseased tissues. The

peptide ligands of the invention can further have valuable diagnostic properties in that they can be used for detecting or identifying the formation of a complex between a labelled compound and other molecules, peptides, proteins, enzymes or receptors. The detecting or identifying methods can use compounds that are labelled with labelling agents such as radioisotopes, enzymes, fluorescent substances, luminous substances (for example, luminol, luminol derivatives, luciferin, aequorin and luciferase), etc. The radioactive isotopes tritium, *i.e.* ^{3}H (T), and carbon-14, i.e. ^{14}C, are particularly useful for this purpose in view of their ease of incorporation and ready means of detection.

Substitution with heavier isotopes such as deuterium, i.e. ^{2}H (D), may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased *in vivo* half-life or reduced dosage requirements, and hence may be preferred in some circumstances.

Substitution with positron emitting isotopes, such as ^{11}C, ^{18}F, ^{15}O and ^{13}N, can be useful in Positron Emission Topography (PET) studies for examining target occupancy.

Isotopically-labelled compounds of peptide ligands of the invention can generally be prepared by conventional techniques known to those skilled in the art or by processes analogous to those described in the accompanying Examples using an appropriate isotopically-labelled reagent in place of the non-labelled reagent previously employed.

Molecular Scaffold

In one embodiment, the molecular scaffold comprises a non-aromatic molecular scaffold. References here in "non-aromatic molecular scaffold" refer to any molecular scaffold as defined herein which does not contain an aromatic (i.e. unsaturated) carbocyclic or heterocyclic ring system.

Suitable examples of non-aromatic molecular scaffolds are described in Heinis *et al.* (2014) Angewandte Chemie, International Edition 53(6) 1602-1606.

As noted in the foregoing documents, the molecular scaffold may be a small molecule, such as a small organic molecule.

In one embodiment the molecular scaffold may be a macromolecule. In one embodiment the molecular scaffold is a macromolecule composed of amino acids, nucleotides or carbohydrates.

In one embodiment the molecular scaffold comprises reactive groups that are capable of reacting with functional group(s) of the polypeptide to form covalent bonds.

The molecular scaffold may comprise chemical groups which form the linkage with a peptide, such as amines, thiols, alcohols, ketones, aldehydes, nitriles, carboxylic acids, esters, alkenes, alkynes, azides, anhydrides, succinimides, maleimides, alkyl halides and acyl halides.

An example of an αβ unsaturated carbonyl containing compound is 1,1',1''-(1,3,5-triazinane-1,3,5-triyl)triprop-2-en-1-one (TATA) (Angewandte Chemie, International Edition (2014), 53(6), 1602-1606).

Multimeric Binding Complexes

According to one aspect of the invention, there is provided a multimeric binding complex which comprises at least two peptide ligands, wherein at least one peptide ligand is specific for P-selectin as defined herein and said peptide ligands may be the same or different, each of which comprises a polypeptide comprising at least three reactive groups, separated by at least two loop sequences, and a molecular scaffold which forms covalent bonds with the reactive groups of the polypeptide such that at least two polypeptide loops are formed on the molecular scaffold.

The present invention describes a series of multimerized bicyclic peptides with various chemical linkers and hinges of various lengths and rigidity using different sites of attachments within said bicyclic peptide which bind and activate targets (such as P-selectin) with a wide range of potency and efficacy.

It will be appreciated by the skilled person that the concept of the invention is the recognition that multiply arranged (multimeric) bicyclic peptides provide a synergistic benefit by virtue of the resultant properties of said multimeric binding complexes compared to the corresponding monomeric binding complexes which contain a single bicyclic peptide. For example, the multimeric binding complexes of the invention typically have greater levels of binding potency or functional activity (as measured herein by EC_{50} values) than their monomeric counterparts. Furthermore, the multimeric binding complexes of the invention are designed to be sufficiently small enough to be cleared by the kidneys.

The complexes of the present invention find particular utility in the treatment of diseases and disorders related to cell adhesion molecules, such as those mediated by cell adhesion molecules. Such diseases and disorders include those of the blood, for example sickle cell disease or sickle cell anaemia and vaso-occlusive crisis. In one embodiment, the disease or disorder is related to sickle cell disease. In another embodiment, the disease or disorder is cancer. In a yet further embodiment, the disease or disorder is cancer metastasis. In one embodiment, the cell adhesion molecule is P-selectin. In a further embodiment, the cell adhesion molecule is E-selectin and/or L-selectin.

In a particular embodiment, at least one of said peptide ligands is specific for P-selectin and at least one of said further peptide ligands binds P-selectin. Thus, in one embodiment, at least two of said peptide ligands bind P-selectin. In a further embodiment, said two or more P-selectin molecules are present on different target cells. In an alternative embodiment, said two or more P-selectin molecules are present on the same target cell.

Thus, in certain embodiments, multimerized peptides of the invention comprise at least two peptide ligands specific for P-selectin. In a further embodiment, multimerized peptides comprise three peptide ligands specific for P-selectin. In another embodiment, the multimerized peptides comprise four peptide ligands specific for P-selectin.

In further embodiments, the cell is selected from a blood cell, such as a red blood cell. In a particular embodiment, the cell is a red blood cell affected by sickle cell disease or sickle cell anaemia. Thus, in one embodiment, the cell is a sickle red blood cell.

Without being bound by theory it is believed that multimerized peptides are able to block cell-surface molecules such as cell adhesion molecules. Thus, in one embodiment, said peptide ligands are specific for the same target. In a further embodiment, the multimeric binding complex comprises at least two identical peptide ligands. By “identical” it is meant peptides having the same amino acid sequence, most critically the same amino acid sequence refers to the binding portion of said peptide (for example, the sequence may vary in attachment position). In this embodiment, each of the peptides within the multimeric binding complex will bind exactly the same epitope upon the same target – the resultant target bound complex will therefore create a homodimer (if the multimeric complex comprises two identical peptides), homotrimer (if the multimeric complex comprises three identical peptides) or homotetramer (if the multimeric complex comprises four identical peptides), etc.

In an alternative embodiment, the multimeric binding complex comprises at least two differing peptide ligands. By "differing" it is meant peptides having a different amino acid sequence. In this embodiment, the differing peptide ligands within the multimeric binding complex will bind to different epitopes on the same target – the resultant target bound complex will therefore create a biparatopic (if the multimeric complex comprises two differing peptides), triparatopic (if the multimeric complex comprises three differing peptides) or tetraparatopic (if the multimeric complex comprises four differing peptides), etc.

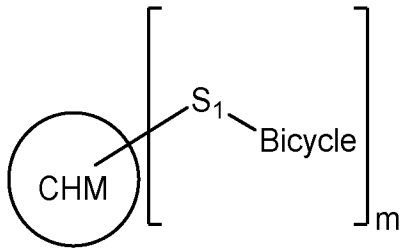
It will be further appreciated that multimerized peptides will be able to block or repress cell surface molecules, such as differing cell adhesion molecules. Thus, in one embodiment, said peptide ligands are specific for different targets. It will be appreciated that in this embodiment, the multimeric binding complex comprises at least two differing peptide ligands (i.e. peptide ligands having differing amino acid sequences). In this embodiment, each of the peptides within the multimeric binding complex will bind a differing epitope upon a different target – the resultant target bound complex will therefore create a bispecific multimeric binding complex (if the multimeric complex comprises two differing peptides), trispecific multimeric binding complex (if the multimeric complex comprises three differing peptides), tetraspecific multimeric binding complex (if the multimeric complex comprises four differing peptides), etc. In a further embodiment, each of the differing epitopes and/or targets are present on the same cell.

It will be appreciated that the multimeric binding complexes of the invention may be designed to be capable of binding to a range of different targets, such as receptors. It will therefore be appreciated that for the bi-, tri- and tetra-specific multimeric binding complexes referred to hereinbefore the peptides may bind to targets on at least two differing cells (such as cells of the blood vessels, endothelial cells or other blood cells, such as other red blood cells).

The peptides within the multimeric binding complexes of the invention may be assembled via a number of differing options. For example, there may be a central hinge or branching moiety with spacer or arm elements radiating from said hinge or branch point each of which will contain a peptide. Alternatively, it could be envisaged that a circular support member may hold a number of inwardly or outwardly projecting peptides.

In one embodiment, each peptide ligand is connected to a central hinge moiety by a spacer group.

It will be appreciated that the spacer group may be linear and connect a single peptide with the central hinge moiety. Thus, in one embodiment, the multimeric binding complex comprises a compound of formula (I):



(I)

wherein CHM represents a central hinge moiety;

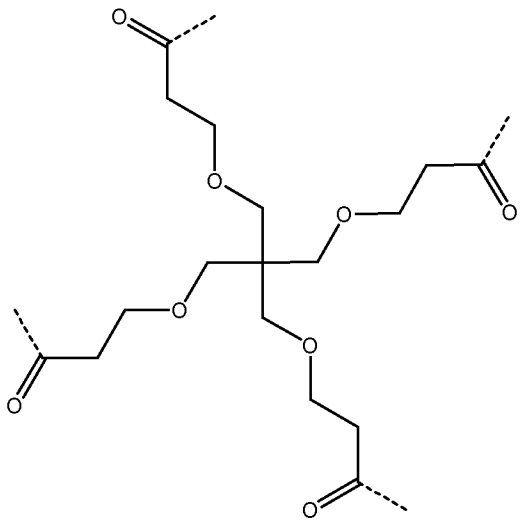
S_1 represents a spacer group;

Bicycle represents a peptide ligand as defined herein; and

m represents an integer selected from 2 to 10.

In one embodiment, m represents an integer selected from 2 to 4. In a further embodiment, m represents an integer selected from 2, 3 or 4.

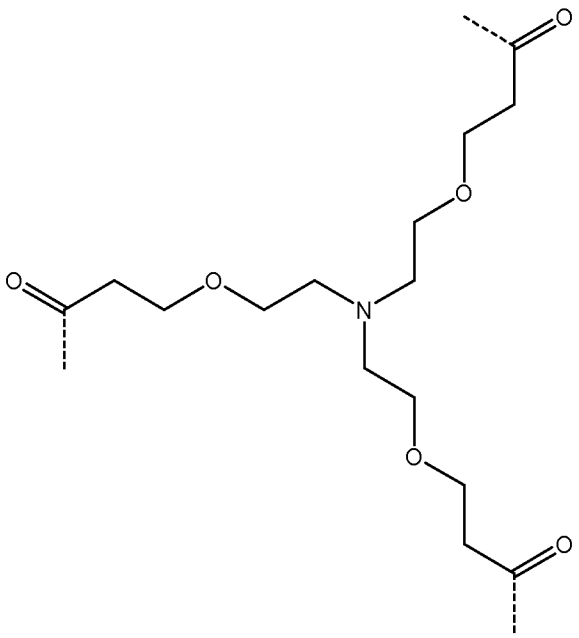
When m represents 4, it will be appreciated that the central hinge moiety will require 4 points of attachment. Thus, in one embodiment, m represents 4 and CHM is a motif of formula (A):



(A)

(referred to herein as Tet), wherein "-----" represents the point of attachment to each S_1 group.

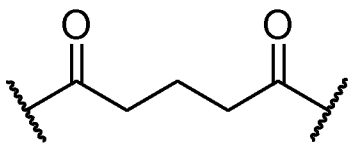
When m represents 3, it will be appreciated that the central hinge moiety will require 3 points of attachment. Thus, in one embodiment, m represents 3 and CHM is a motif of formula (B):



(B)

(referred to herein as TCA), wherein "-----" represents the point of attachment to each S_1 group.

When m represents 2, it will be appreciated that the central hinge moiety will require 2 points of attachment. Thus, in one embodiment, m represents 2 and CHM is a motif of formula (C):



(C)

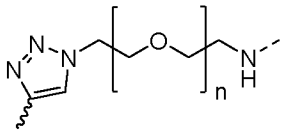
(referred to herein as GTA), wherein " ~~~ " represents the point of attachment to each S_1 group.

It will be readily apparent to the skilled person how alternative central hinge moieties may be constructed depending upon the value of m.

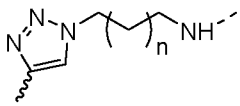
It will be appreciated that the spacer (S_1) may be any suitable construction to link the peptide central hinge moiety to the peptide. In one embodiment, the spacer (S_1) comprises a triazolyl moiety. The advantage of this embodiment is that the triazolyl moiety may be incorporated within the synthesis using commonly available "click" chemistry. Examples of suitable spacer (S_1) groups include one or more PEG moieties, peptide sequences, carbohydrates, lipids and the like.

In a further embodiment, the spacer (S_1) comprises one or more PEG moieties. References herein to "PEG" refer to a linear polymer with a regular repeat unit of the general structure: $(CH_2CH_2O)_n$- (where n represents any number, such as 1 to 30).

Thus, in a further embodiment, the spacer (S_1) is selected from any one of spacers S_1A, S_1B, S_1C, S_1D, S_1E, S_1F, S_1G and S_1H:

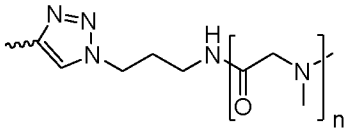


S_1A
n = 5, 10 and 23

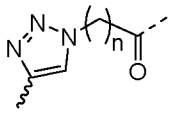


S_1E

n =1

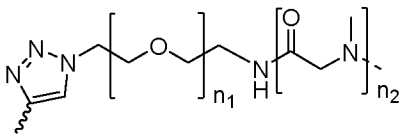


S_1B
n = 5, 10



S_1F

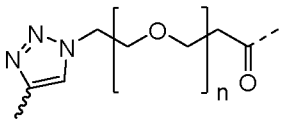
n =1



S_1C

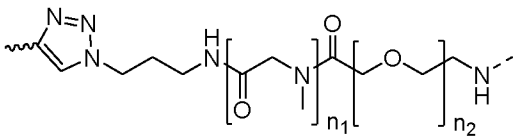
n_1 =5, n_2 =5

n_1 =10, n_2 =10



S_1G

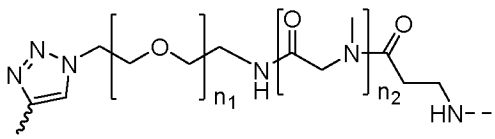
n = 5 and 10



S_1D

n_1 =5, n_2 =5

n_1 =10, n_2 =10



S_1H

n_1 =5, n_2 =5

n_1 =10, n_2 =10

wherein "-----" represents the point of attachment to the CHM group; and

" ~~~ " represents the point of attachment to the Bicycle group.

In a yet further embodiment, the spacer (S_1) is S_1A. In a further embodiment, n represents 5, 10 or 23.

Tetrameric Binding Complexes

In one embodiment, the multimeric binding complex comprises a tetrameric binding complex described in the following Tables 1A and 1B:

Table 1A: Exemplified Tetrameric Binding Complexes of the Invention

Multimer Compound Number	Corresponding Monomer	Number of Monomers	Central Hinge Moiety	Spacer Molecule	Attachment Point
BCY5462	BCY10910	4	A (Tet)	S_1A: n = 10	N-terminal Pya
BCY5463	BCY10910	4	A (Tet)	S_1A: n = 23	N-terminal Pya
BCY5464	BCY10911	4	A (Tet)	S_1A: n = 10	C-terminal K(Pya)
BCY5465	BCY10911	4	A (Tet)	S_1A: n = 23	C-terminal K(Pya)
BCY12261	BCY12025	4	A (Tet)	S_1A: n = 23	C-terminal K(Pya)
BCY12262	BCY12026	4	A (Tet)	S_1A: n = 23	C-terminal K(Pya)
BCY11903	BCY11890	4	A (Tet)	S_1A: n = 23	C-terminal K(Pya)
BCY19238	BCY18041	4	A (Tet)	S_1A: n = 23	C-terminal K(Pya)

In one embodiment, the multimeric binding complex comprises a tetrameric binding complex which is other than BCY11903 and/or BCY19238.

Table 1B: Preferred Exemplified Tetrameric Binding Complexes of the Invention

Multimer Compound Number	Corresponding Monomer	Number of Monomers	Central Hinge Moiety	Spacer Molecule	Attachment Point
BCY5462	BCY10910	4	A (Tet)	S_1A: n = 10	N-terminal Pya
BCY5463	BCY10910	4	A (Tet)	S_1A: n = 23	N-terminal Pya
BCY5464	BCY10911	4	A (Tet)	S_1A: n = 10	C-terminal K(Pya)
BCY5465	BCY10911	4	A (Tet)	S_1A: n = 23	C-terminal K(Pya)

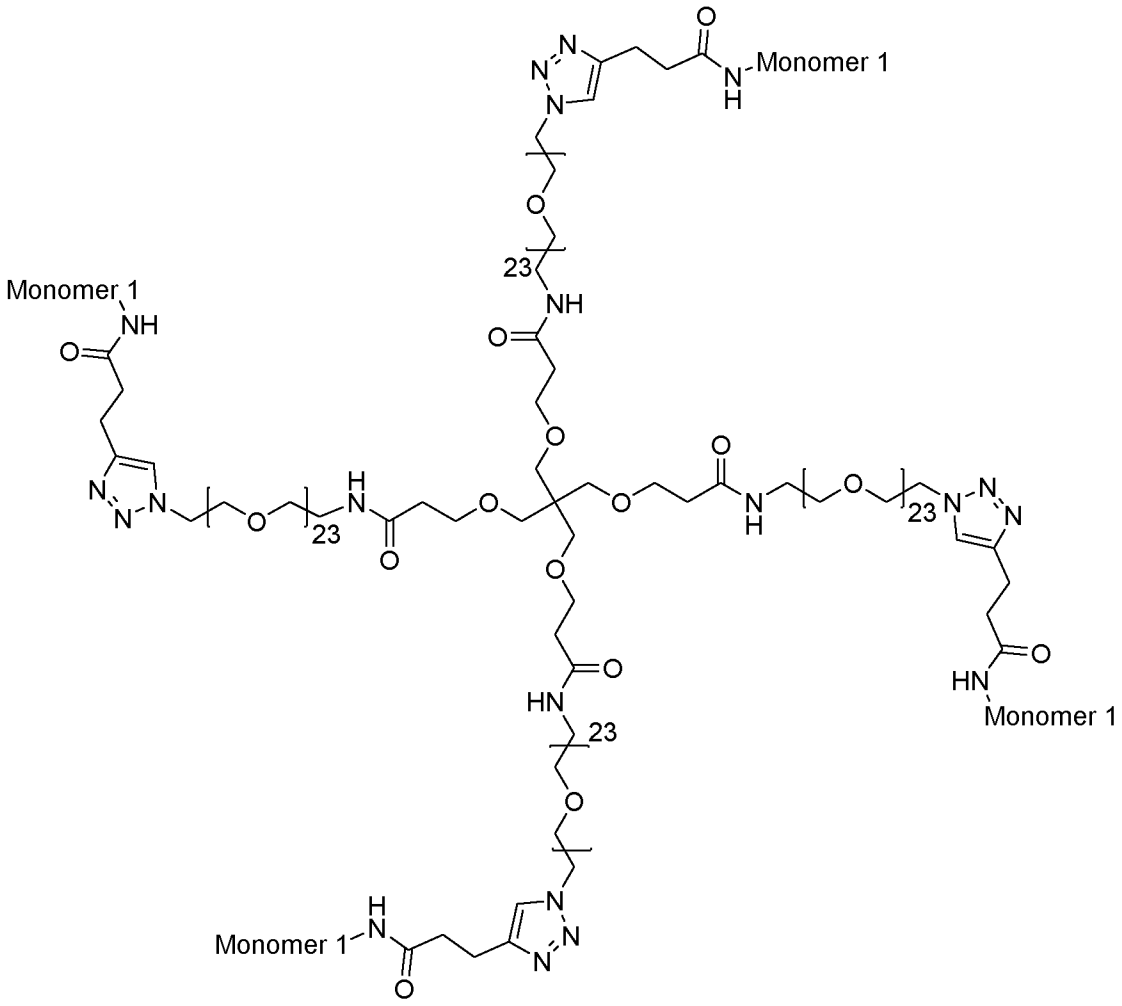
BCY12261	BCY12025	4	A (Tet)	S_1A: n = 23	C-terminal K(Pya)
BCY12262	BCY12026	4	A (Tet)	S_1A: n = 23	C-terminal K(Pya)

Data is presented herein which demonstrates that the tetrameric binding complexes of Table 1B displayed good binding to P-selectin, with affinity K_D values of less than 100nM or less than 0.02nM (see Table 6). In particular, data is presented herein in Figure 2 where it can be seen that significant inhibition of white blood cell adhesion was observed for BCY12262 that compares well to the positive control antibody (crizanlizumab).

In one embodiment, the multimeric binding complex comprises a tetramer comprising four bicyclic peptides each of which are BCY10910 as defined herein, which are linked via the N-terminal Pya moiety to a spacer molecule (S_1A) wherein n represents 10 or 23 and wherein (S_1A) is linked to a central hinge moiety which is (A) as defined herein. The multimeric binding complexes according to this embodiment are referred to herein as BCY5462 and BCY5463, respectively.

In a further embodiment, the multimeric binding complex comprises a tetramer comprising four bicyclic peptides each of which are BCY10911 as defined herein, which are linked via the C-terminal Lys(Pya) moiety to a spacer molecule (S_1A) wherein n represents 10 or 23 and wherein (S_1A) is linked to a central hinge moiety which is (A) as defined herein. The multimeric binding complexes according to this embodiment are referred to herein as BCY5464 and BCY5465, respectively.

In one embodiment, the multimeric binding complex comprises a tetramer comprising four bicyclic peptides each of which are BCY12025 or BCY12026 as defined herein, which are linked via the C-terminal Lys(Pya) moiety to a spacer molecule (S_1A) wherein n represents 23 and wherein (S_1A) is linked to a central hinge moiety which is (A) as defined herein. These multimeric binding complexes are referred to herein as BCY12261 and BCY12262, respectively:

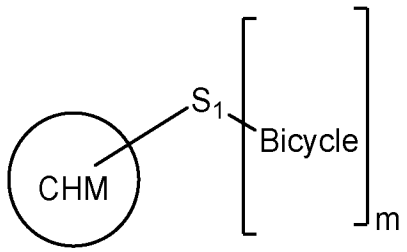


BCY12261 or BCY12262,

wherein Monomer 1 represents BCY12025 or BCY12026 respectively.

Data is presented herein in Table 6 which shows that BCY12261 and BCY12262 bind P-selectin with affinity K_D values of 0.019nM and 0.017nM respectively.

In an alternative arrangement the spacer group may be branched and thus a single spacer group may connect multiple peptides with the central hinge moiety. Thus, in an alternative embodiment, the multimeric binding complex comprises a compound of formula (II):



(II)

wherein CHM represents a central hinge moiety;

S_1 represents a spacer group;
Bicycle represents a peptide ligand as defined herein; and
m represents an integer selected from 2 to 10.

It will be appreciated that the peptide ligand may be attached to the spacer via a number of means. In one embodiment, the peptide ligand is conjugated to one half of a binding pair and said other half of said binding pair links each of the peptides to the spacer.

In one embodiment, said binding pair comprises biotin and streptavidin. Thus, each peptide ligand is conjugated to biotin and linked to the spacer via streptavidin.

Trimeric Binding Complexes

In one embodiment, the multimeric binding complex comprises a trimeric binding complex described in the following Tables 2A and 2B:

Table 2A: Exemplified Trimeric Binding Complexes of the Invention

Multimer Compound Number	**Corresponding Monomer**	**Number of Monomers**	**Central Hinge Moiety**	**Spacer Molecule**	**Attachment Point**
BCY5458	BCY10910	3	B (TCA)	S_1A: n = 10	N-terminal Pya
BCY5459	BCY10910	3	B (TCA)	S_1A: n = 23	N-terminal Pya
BCY5460	BCY10911	3	B (TCA)	S_1A: n = 10	C-terminal K(Pya)
BCY5461	BCY10911	3	B (TCA)	S_1A: n = 23	C-terminal K(Pya)
BCY12259	BCY12025	3	B (TCA)	S_1A: n = 23	C-terminal K(Pya)
BCY12260	BCY12026	3	B (TCA)	S_1A: n = 23	C-terminal K(Pya)
BCY19242	BCY11890	3	B (TCA)	S_1A: n = 23	C-terminal K(Pya)
BCY19239	BCY18041	3	B (TCA)	S_1A: n = 23	C-terminal K(Pya)

In one embodiment, the multimeric binding complex comprises a trimeric binding complex which is other than BCY19242 and/or BCY19239.

Table 2B: Preferred Exemplified Trimeric Binding Complexes of the Invention

Multimer Compound Number	Corresponding Monomer	Number of Monomers	Central Hinge Moiety	Spacer Molecule	Attachment Point
BCY5458	BCY10910	3	B (TCA)	S_1A: n = 10	N-terminal Pya
BCY5459	BCY10910	3	B (TCA)	S_1A: n = 23	N-terminal Pya
BCY5460	BCY10911	3	B (TCA)	S_1A: n = 10	C-terminal K(Pya)
BCY5461	BCY10911	3	B (TCA)	S_1A: n = 23	C-terminal K(Pya)
BCY12259	BCY12025	3	B (TCA)	S_1A: n = 23	C-terminal K(Pya)
BCY12260	BCY12026	3	B (TCA)	S_1A: n = 23	C-terminal K(Pya)

Data is presented herein which demonstrates that the tetrameric binding complexes of Table 2B displayed good binding to P-selectin, with affinity K_D values of less than 500nM or less than 0.5nM (see Table 6).

In one embodiment, the multimeric binding complex comprises a trimer comprising three bicyclic peptides each of which are BCY10910 as defined herein, which are linked via the N-terminal Pya moiety to a spacer molecule (S_1A) wherein n represents 10 or 23 and wherein (S_1A) is linked to a central hinge moiety which is (B) as defined herein. The multimeric binding complexes according to this embodiment are referred to herein as BCY5458 and BCY5459, respectively.

In a further embodiment, the multimeric binding complex comprises a trimer comprising three bicyclic peptides each of which are BCY10911 as defined herein, which are linked via the C-terminal Lys(Pya) moiety to a spacer molecule (S_1A) wherein n represents 10 or 23 and wherein (S_1A) is linked to a central hinge moiety which is (B) as defined herein. The multimeric binding complexes according to this embodiment are referred to herein as BCY5460 and BCY5461, respectively.

In one embodiment, the multimeric binding complex comprises a trimer comprising three bicyclic peptides each of which are BCY12025 or BCY12026 as defined herein, which are linked via the C-terminal Lys(Pya) moiety to a spacer molecule (S_1A) wherein n represents 23 and wherein (S_1A) is linked to a central hinge moiety which is (B) as defined herein. These multimeric binding complexes are referred to herein as BCY12259 and BCY12260, respectively.

Data is presented herein in Table 6 which shows that BCY12259 and BCY12260 bind P-selectin with affinity K_D values of 0.233nM and 0.109nM respectively.

Tandem Binding Complexes

In one embodiment, the multimeric binding complex comprises a tandem binding complex described in the following Tables 3A and 3B:

Table 3A: Exemplified Tandem Binding Complexes of the Invention

Multimer Compound Number	**Corresponding Monomer**	**Number of Monomers**	**Central Hinge Moiety**	**Spacer Molecule**	**Attachment Point**
BCY5454	BCY10910	2	C (GTA)	S_1A: n = 10	N-terminal Pya
BCY5455	BCY10910	2	C (GTA)	S_1A: n = 23	N-terminal Pya
BCY5456	BCY10911	2	C (GTA)	S_1A: n = 10	C-terminal K(Pya)
BCY5457	BCY10911	2	C (GTA)	S_1A: n = 23	C-terminal K(Pya)
BCY12257	BCY12025	2	C (GTA)	S_1A: n = 23	C-terminal K(Pya)
BCY12258	BCY12026	2	C (GTA)	S_1A: n = 23	C-terminal K(Pya)
BCY19243	BCY11890	2	C (GTA)	S_1A: n = 23	C-terminal K(Pya)
BCY19240	BCY18041	2	C (GTA)	S_1A: n = 23	C-terminal K(Pya)

In one embodiment, the multimeric binding complex comprises a dimeric binding complex which is other than BCY19243 and/or BCY19240.

Table 3B: Preferred Exemplified Tandem Binding Complexes of the Invention

Multimer Compound Number	Corresponding Monomer	Number of Monomers	Central Hinge Moiety	Spacer Molecule	Attachment Point
BCY5454	BCY10910	2	C (GTA)	S_1A: n = 10	N-terminal Pya
BCY5455	BCY10910	2	C (GTA)	S_1A: n = 23	N-terminal Pya
BCY5456	BCY10911	2	C (GTA)	S_1A: n = 10	C-terminal K(Pya)
BCY5457	BCY10911	2	C (GTA)	S_1A: n = 23	C-terminal K(Pya)
BCY12257	BCY12025	2	C (GTA)	S_1A: n = 23	C-terminal K(Pya)
BCY12258	BCY12026	2	C (GTA)	S_1A: n = 23	C-terminal K(Pya)

Data is presented herein which demonstrates that the dimeric binding complexes of Table 3B displayed good binding to P-selectin, with affinity K_D values of about 1200nM, less than 1000nM, less than 500nM or less than 0.5nM (see Table 6).

In one embodiment, the multimeric binding complex comprises a tandem comprising two bicyclic peptides each of which are BCY10910 as defined herein, which are linked via the N-terminal PYA moiety to a spacer molecule (S_1A) wherein n represents 10 or 23 and wherein (S_1A) is linked to a central hinge moiety which is (C) as defined herein. The multimeric binding complexes according to this embodiment are referred to herein as BCY5454 and BCY5455, respectively.

In a further embodiment, the multimeric binding complex comprises a tandem comprising two bicyclic peptides each of which are BCY10911 as defined herein, which are linked via the C-terminal Lys(Pya) moiety to a spacer molecule (S_1A) wherein n represents 10 or 23 and wherein (S_1A) is linked to a central hinge moiety which is (C) as defined herein. The multimeric binding complexes according to this embodiment are referred to herein as BCY5456 and BCY5457, respectively.

In one embodiment, the multimeric binding complex comprises a tandem comprising two bicyclic peptides each of which are BCY12025 or BCY12026 as defined herein, which are linked via the C-terminal Lys(Pya) moiety to a spacer molecule (S_1A) wherein n represents 23

and wherein (S_1A) is linked to a central hinge moiety which is (C) as defined herein. These multimeric binding complexes are referred to herein as BCY12257 and BCY12258, respectively.

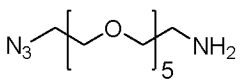
Data is presented herein in Table 6 which shows that BCY12257 and BCY12258 bind P-selectin with affinity K_D values of 11.6nM and 0.409nM respectively.

Linkers

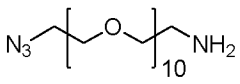
It will be appreciated that the P-selectin peptide ligand may be conjugated to the second peptide ligand via any suitable linker. Typically the design of said linker will be such that the two Bicyclic peptides are presented in such a manner that they can bind unencumbered to their respective targets either alone or while simultaneously binding to both target receptors. Additionally, the linker should permit binding to both targets simultaneously while maintaining an appropriate distance between the target cells or receptors that would lead to the desired functional outcome. The properties of the linker may be modulated to increase length, rigidity or solubility to optimise the desired functional outcome. The linker may also be designed to permit the attachment of more than one Bicycle to the same target. Increasing the valency of either binding peptide may serve to increase the affinity of the complex for the target cells or may help to block one or both of the target cell surface molecules.

In one embodiment, the linker is selected from the following sequences: -CH_2-, -PEG_5-, -PEG_{10}-, -PEG_{12}-, -PEG_{23}-, -PEG_{24}-, -PEG_{15}-Sar_5-, -PEG_{10}-Sar_{10}-, -PEG_5-Sar_{15}-, -PEG_5-Sar_5-, -B-Ala-Sar_{20}-, -B-Ala-Sar_{10}-PEG_{10}-, -B-Ala-Sar_5-PEG_{15}- and -B-Ala-Sar_5-PEG_5-.

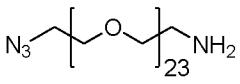
Structural representations of suitable linkers are detailed below:



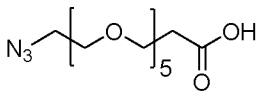
H2N-Peg5-N3
COM00000132



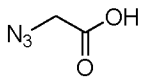
H2N-Peg10-N3
COM00000134



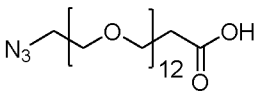
H2N-Peg23-N3
COM00000135



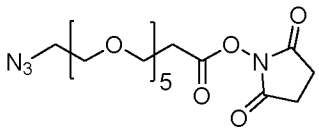
N3-PEG5-COOH
COM00000467



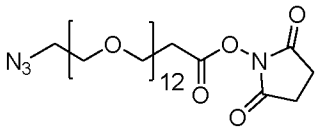
N3-CH2-COOH
COM00000468



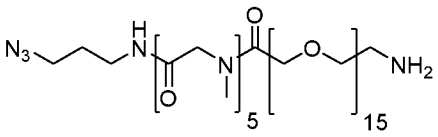
N3-PEG12-COOH
COM00000466



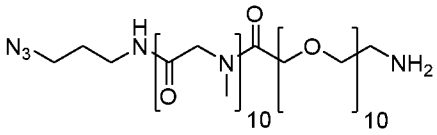
NHS-PEG5-N3



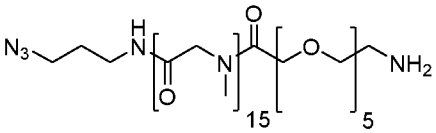
NHS-PEG12-N3



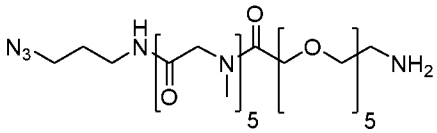
H2N-PEG15-SAR5-N3
COM00000128



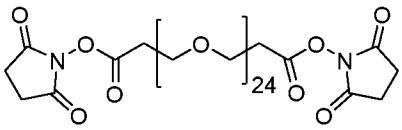
H2N-PEG10-SAR10-N3
COM00000129



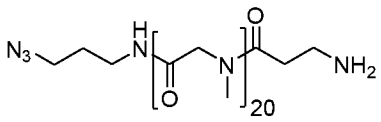
H2N-PEG5-SAR15-N3
COM00000130



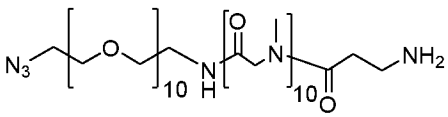
H2N-PEG5-SAR5-N3
COM00000131



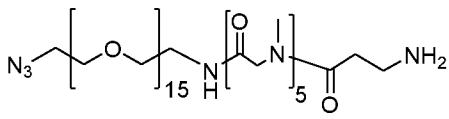
NHS-PEG24-NHS
COM00000469



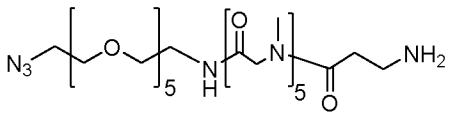
H2N-(B-Ala)-SAR20-N3
COM00000470



H2N-(B-Ala)-SAR10-PEG10-N3
COM00000471



H2N-(B-Ala)-SAR5-PEG15-N3
COM00000472



H2N-(B-Ala)-SAR5-PEG5-N3

COM00000473

Synthesis

The peptides of the present invention may be manufactured synthetically by standard techniques followed by reaction with a molecular scaffold *in vitro*. When this is performed, standard chemistry may be used. This enables the rapid large scale preparation of soluble material for further downstream experiments or validation. Such methods could be accomplished using conventional chemistry such as that disclosed in Timmerman *et al.* (*supra*).

Thus, the invention also relates to the manufacture of polypeptides or conjugates selected as set out herein, wherein the manufacture comprises optional further steps as explained below. In one embodiment, these steps are carried out on the end product polypeptide/conjugate made by chemical synthesis.

Optionally amino acid residues in the polypeptide of interest may be substituted when manufacturing a conjugate or complex.

Peptides can also be extended, to incorporate for example another loop and therefore introduce multiple specificities.

To extend the peptide, it may simply be extended chemically at its N-terminus or C-terminus or within the loops using orthogonally protected lysines (and analogues) using standard solid phase or solution phase chemistry. Standard (bio)conjugation techniques may be used to introduce an activated or activatable N- or C-terminus. Alternatively additions may be made by fragment condensation or native chemical ligation e.g. as described in (Dawson *et al.* 1994. Synthesis of Proteins by Native Chemical Ligation. Science 266:776-779), or by enzymes, for example using subtiligase as described in (Chang *et al.* Proc Natl Acad Sci U S A. 1994 Dec 20; 91(26):12544-8 or in Hikari *et al.* Bioorganic & Medicinal Chemistry Letters Volume 18, Issue 22, 15 November 2008, Pages 6000-6003).

Alternatively, the peptides may be extended or modified by further conjugation through disulphide bonds. This has the additional advantage of allowing the first and second peptide

to dissociate from each other once within the reducing environment of the cell. In this case, the molecular scaffold (e.g. TATA or TCAZ) could be added during the chemical synthesis of the first peptide so as to react with the three cysteine groups; a further cysteine or thiol could then be appended to the N- or C-terminus of the first peptide, so that this cysteine or thiol only reacted with a free cysteine or thiol of the second peptide, forming a disulphide-linked bicyclic peptide-peptide conjugate.

Similar techniques apply equally to the synthesis/coupling of two bicyclic and bispecific macrocycles, potentially creating a tetraspecific molecule.

Furthermore, addition of other functional groups or effector groups may be accomplished in the same manner, using appropriate chemistry, coupling at the N- or C-termini or via side chains. In one embodiment, the coupling is conducted in such a manner that it does not block the activity of either entity.

Drug Conjugates

According to a further aspect of the invention, there is provided a drug conjugate comprising a peptide ligand or multimeric binding complex as defined herein conjugated to one or more effector and/or functional groups.

Effector and/or functional groups can be attached, for example, to the N- and/or C-termini of the polypeptide, to an amino acid within the polypeptide, or to the molecular scaffold.

Appropriate effector groups include antibodies and parts or fragments thereof. For instance, an effector group can include an antibody light chain constant region (CL), an antibody CH1 heavy chain domain, an antibody CH2 heavy chain domain, an antibody CH3 heavy chain domain, or any combination thereof, in addition to the one or more constant region domains. An effector group may also comprise a hinge region of an antibody (such a region normally being found between the CH1 and CH2 domains of an IgG molecule).

In a further embodiment of this aspect of the invention, an effector group according to the present invention is an Fc region of an IgG molecule. Advantageously, a peptide ligand-effector group according to the present invention comprises or consists of a peptide ligand Fc fusion having a tβ half-life of a day or more, two days or more, 3 days or more, 4 days or more, 5 days or more, 6 days or more or 7 days or more. Most advantageously, the peptide ligand according to the present invention comprises or consists of a peptide ligand Fc fusion having a half-life of a day or more.

Functional groups include, in general, binding groups, drugs, reactive groups for the attachment of other entities, functional groups which aid uptake of the macrocyclic peptides into cells, and the like.

The ability of peptides to penetrate into cells will allow peptides against intracellular targets to be effective. Targets that can be accessed by peptides with the ability to penetrate into cells include transcription factors, intracellular signalling molecules such as tyrosine kinases and molecules involved in the apoptotic pathway. Functional groups which enable the penetration of cells include peptides or chemical groups which have been added either to the peptide or the molecular scaffold. Peptides such as those derived from such as VP22, HIV-Tat, a homeobox protein of Drosophila (Antennapedia), e.g. as described in Chen and Harrison, Biochemical Society Transactions (2007) Volume 35, part 4, p821; Gupta *et al.* in Advanced Drug Discovery Reviews (2004) Volume 57 9637. Examples of short peptides which have been shown to be efficient at translocation through plasma membranes include the 16 amino acid penetratin peptide from Drosophila Antennapedia protein (Derossi *et al.* (1994) J Biol. Chem. Volume 269 p10444), the 18 amino acid 'model amphipathic peptide' (Oehlke *et al.* (1998) Biochim Biophys Acts Volume 1414 p127) and arginine rich regions of the HIV TAT protein. Non peptidic approaches include the use of small molecule mimics or SMOCs that can be easily attached to biomolecules (Okuyama *et al.* (2007) Nature Methods Volume 4 p153). Other chemical strategies to add guanidinium groups to molecules also enhance cell penetration (Elson-Scwab *et al.* (2007) J Biol Chem Volume 282 p13585). Small molecular weight molecules such as steroids may be added to the molecular scaffold to enhance uptake into cells.

One class of functional groups which may be attached to peptide ligands includes antibodies and binding fragments thereof, such as Fab, Fv or single domain fragments. In particular, antibodies which bind to proteins capable of increasing the half-life of the peptide ligand *in vivo* may be used.

In one embodiment, a peptide ligand-effector group according to the invention has a tβ half-life selected from the group consisting of: 12 hours or more, 24 hours or more, 2 days or more, 3 days or more, 4 days or more, 5 days or more, 6 days or more, 7 days or more, 8 days or more, 9 days or more, 10 days or more, 11 days or more, 12 days or more, 13 days or more, 14 days or more, 15 days or more or 20 days or more. Advantageously a peptide ligand-effector group or composition according to the invention will have a tβ half-life in the range 12

to 60 hours. In a further embodiment, it will have a tβ half-life of a day or more. In a further embodiment still, it will be in the range 12 to 26 hours.

In one particular embodiment of the invention, the functional group is selected from a metal chelator, which is suitable for complexing metal radioisotopes of medicinal relevance.

Possible effector groups also include enzymes, for instance such as carboxypeptidase G2 for use in enzyme/prodrug therapy, where the peptide ligand replaces antibodies in ADEPT.

In one embodiment, the multimeric binding complexes of the invention contain a cleavable bond, such as a disulphide bond or a protease sensitive bond. Without being bound by theory it is believed that such a cleavable moiety deactivates the complex until it reaches the tumour microenvironment. The benefit of this embodiment provides for the complex to be reduced in size following binding to the target. In a further embodiment, the groups adjacent to the disulphide bond are modified to control the hindrance of the disulphide bond, and by this the rate of cleavage and concomitant release of the binding agent.

Published work established the potential for modifying the susceptibility of the disulphide bond to reduction by introducing steric hindrance on either side of the disulphide bond (Kellogg *et al.* (2011) Bioconjugate Chemistry, 22, 717). A greater degree of steric hindrance reduces the rate of reduction by intracellular glutathione and also extracellular (systemic) reducing agents, consequentially reducing the ease by which toxin is released, both inside and outside the cell. Thus, selection of the optimum in disulphide stability in the circulation (which minimises undesirable side effects of the toxin) versus efficient release in the intracellular milieu (which maximises the therapeutic effect) can be achieved by careful selection of the degree of hindrance on either side of the disulphide bond.

The hindrance on either side of the disulphide bond is modulated through introducing one or more methyl groups on the targeting entity (here, the bicyclic peptide ligand).

Pharmaceutical Compositions

According to a further aspect of the invention, there is provided a pharmaceutical composition comprising a peptide ligand, multimeric binding complex or drug conjugate as defined herein in combination with one or more pharmaceutically acceptable excipients.

Generally, the present peptide ligands will be utilised in purified form together with pharmacologically appropriate excipients or carriers. Typically, these excipients or carriers

include aqueous or alcoholic/aqueous solutions, emulsions or suspensions, including saline and/or buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride and lactated Ringer's. Suitable physiologically-acceptable adjuvants, if necessary to keep a polypeptide complex in suspension, may be chosen from thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin and alginates.

Intravenous vehicles include fluid and nutrient replenishers and electrolyte replenishers, such as those based on Ringer's dextrose. Preservatives and other additives, such as antimicrobials, antioxidants, chelating agents and inert gases, may also be present (Mack (1982) Remington's Pharmaceutical Sciences, 16th Edition).

The peptide ligands of the present invention may be used as separately administered compositions or in conjunction with other agents. These can include antibodies, antibody fragments and various immunotherapeutic drugs, such as cyclosporine, methotrexate, adriamycin or cisplatinum and immunotoxins. Further examples of other agents which may be administered separately or in conjunction with the peptide ligands of the invention include cytokines, lymphokines, other hematopoietic factors, thrombolytic and anti-thrombotic factors. Pharmaceutical compositions can include "cocktails" of various cytotoxic or other agents in conjunction with the protein ligands of the present invention, or even combinations of selected polypeptides according to the present invention having different specificities, such as polypeptides selected using different target ligands, whether or not they are pooled prior to administration.

The route of administration of pharmaceutical compositions according to the invention may be any of those commonly known to those of ordinary skill in the art. For therapy, the peptide ligands of the invention can be administered to any patient in accordance with standard techniques. The administration can be by any appropriate mode, including parenterally, intravenously, intramuscularly, intraperitoneally, transdermally, via the pulmonary route, or also, appropriately, by direct infusion with a catheter. Preferably, the pharmaceutical compositions according to the invention will be administered intravenously. The dosage and frequency of administration will depend on the age, sex and condition of the patient, concurrent administration of other drugs, counterindications and other parameters to be taken into account by the clinician.

The peptide ligands of this invention can be lyophilised for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective and art-known

lyophilisation and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilisation and reconstitution can lead to varying degrees of activity loss and that levels may have to be adjusted upward to compensate.

The compositions containing the present peptide ligands or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In certain therapeutic applications, an adequate amount to accomplish at least partial inhibition, suppression, modulation, killing, or some other measurable parameter, of a population of selected cells is defined as a "therapeutically-effective dose". Amounts needed to achieve this dosage will depend upon the severity of the disease, but generally range from 0.005 to 5.0 mg of selected peptide ligand per kilogram of body weight, with doses of 0.05 to 2.0 mg/kg/dose being more commonly used. For prophylactic applications, compositions containing the present peptide ligands or cocktails thereof may also be administered in similar or slightly lower dosages.

A composition containing a peptide ligand according to the present invention may be utilised in prophylactic and therapeutic settings to aid in the alteration, inactivation, killing or removal of a select target cell population in a mammal. In addition, the peptide ligands described herein may be used extracorporeally or *in vitro* selectively to kill, deplete or otherwise effectively remove a target cell population from a heterogeneous collection of cells. Blood from a mammal may be combined extracorporeally with the selected peptide ligands whereby the undesired cells are killed or otherwise removed from the blood for return to the mammal in accordance with standard techniques.

Therapeutic Uses

The bicyclic peptides of the invention have specific utility as P-selectin binding agents. According to a further aspect of the invention, there is provided a peptide ligand, multimeric binding complex, drug conjugate or pharmaceutical composition as defined herein for use in preventing, suppressing or treating a disease or disorder mediated cell adhesion molecules, such as P-selectin, E-selectin and L-selectin.

P-selectin is a cell adhesion molecule expressed on the surface of activated endothelial cells and platelets. In unactivated endothelial cells and platelets, P-selectin is stored in granules. P-selectin is involved in the recruitment of white blood cells during inflammation and the aggregation of platelets during vascular injury. Activation of platelets by thrombin results in "membrane flipping" where α-granules and dense granules are released, exposing their inner walls to the outside of the cell. Platelet aggregation is then promoted by P-selectin through platelet-fibrin and platelet-platelet binding.

P-selectin has also been shown to have a role in tumour metastasis, similar to E-selectin (Kohler *et al.,* British Journal of Cancer 2010, 102:3). The expression of P-selectin on the surface of activated endothelial cells and platelets also aids the invasion of cancer cells into the blood stream. The formation of complexes of activated platelets with tumour cells in the vasculature prevents the recognition of tumour cells by macrophages, shielding them from recognition by the immune system. It has been shown that a reduction in circulating platelets could reduce cancer metastasis (Gasic GL, Cancer Metastasis Reviews 1984, 3:2). Furthermore, the related cell adhesion molecule, E-selectin, has been shown to be constitutively expressed on the surface of bone marrow endothelium, indicating a route by which tumour cells may metastasise preferentially to the bone.

The role of P-selectin in cardiovascular conditions such as cardiac inflammation and fibrosis, coronary heart disease, hypertension and atrial fibrillation has also been reported (Liu *et al.*, Molecular Medicine Reports 2016, 13(6) and Blann *et al.*, European Heart Journal 2003, 24(24)). For example, increased P-selectin expression has been demonstrated on active atherosclerotic plaques and animals lacking P-selectin have been seen to have decreased tendency to form atherosclerotic plaques. Furthermore, the P-selectin antagonist, inclacumab reduces myocardial damage after percutaneous coronary intervention in non–ST-segment elevation myocardial infarction patients and platelet-leukocyte aggregates were inhibited (Stahli *et al.*, Journal of the American Heart Association 2016, 16:5(11) and Schmitt *et al.*, Jornal of Cardiovascular Pharmacology 2015, 65(6)). Therefore, the antagonism or blocking of P-selectin will likely find therapeutic benefit.

In one embodiment, the P-selectin is mammalian P-selectin. In a further embodiment, the mammalian P-selectin is human P-selectin.

P-selectin peptides will be primarily (but not exclusively) used to antagonistically block P-selectin or a related cell adhesion molecule (e.g. E-selectin and/or L-selectin), and consequently activated endothelial cells and platelets. It will be appreciated that such antagonism will find particular utility in the prevention, suppression and/or treatment of vaso-occlusive crisis or other disease/disorder related to sickle cell disease or sickle cell anaemia.

P-selectin peptides may also be used to antagonistically block P-selectin or a related cell adhesion molecule (e.g. E-selectin and/or L-selectin) binding to glycoproteins or glycolipid ligands expressed on a cancer cell or cancer, and consequently the metastasis or tumour invasion of said cancer cell or cancer. Such cancers include, but are not limited to early or

late stage human malignancies, which include solid tumours such as Non-Small Cell Lung Carcinomas (NSCLC), breast cancers, including triple negative breast cancers (TNBC), ovarian cancers, prostate cancers, bladder cancers, urothelial carcinomas, colorectal cancers, head and neck cancer, Squamous Cell Carcinoma of the Head and Neck (SCCHN), melanomas, pancreatic cancers, and other advanced solid tumours. The cancer may also be selected from a blood cancer.

The term "solid tumour" as used herein is defined as an abnormal growth of tissues without much liquid mass in it, while non-solid tumours are generally dispersed cancers without any or significant solid masses. Examples of solid tumours are carcinomas, sarcomas and lymphomas. Blood cancers (leukaemias) such as acute myeloid leukaemia (AML), Acute lymphocytic (or lymphoblastic) leukaemia (ALL) are non-solid tumours. P-selectin peptides or the complexes defined herein may be used as a monotherapy agent in the aforementioned cancer indications to block, prevent or reduce tumour cell metastasis. In addition to use as a monotherapy agent in cancer, P-selectin peptides, as well as complexes and conjugates thereof as defined herein, may be used in combination with immunotherapy agents, such as anti-PD-1 and anti-CTLA4 agents. Additional therapeutic applications of P-selectin peptides, complexes and conjugates thereof include, but are not restricted to, mono or combination therapies with radiation cancer treatments, and cancer vaccines. Non-cancerous therapeutic applications of P-selectin peptides either as monotherapy or in combination therapy, include but are not limited to, diseases and disorders related to sickle cell disease/anaemia, such as vaso-occlusive crisis.

It will be appreciated that P-selectin peptides may be useful in the treatment of conditions characterised by intercellular adhesion mediated by P-selectin or a related cell adhesion molecule (e.g. E-selectin and/or L-selectin). Such conditions include, without limitation: post-ischemic leukocyte-mediated tissue damage (reperfusion injury), such as that related to myocardial infarction or transplant; myocardial infarction; bacterial or viral infection; metastatic conditions; inflammatory disorders, such as arthritis; acute respiratory distress syndrome (ARDS); asthma; emphysema; delayed type hypersensitivity reaction; systemic lupus erythematosus (SLE); thermal injury; autoimmune thyroiditis; experimental allergic encephalomyelitis (EAE); multiple sclerosis (MS); multiple organ injury syndrome secondary to trauma; diabetes; Reynaud's syndrome; neutrophilic dermatosis (Sweet's syndrome); inflammatory bowel disease (IBD); Grave's disease; glomerulonephritis; gingivitis; periodontitis; haemolytic uremic syndrome; ulcerative colitis; Crohn's disease; necrotizing enterocolitis; granulocyte transfusion associated syndrome; and cytokine-induced toxicity. Further examples of conditions or treatments in which P-selectin peptides may find utility

include: organ transplantation, including to prepare organs prior to transplantation and to reduce transplant rejection; haemodialysis; and leukapheresis.

Thus, in one embodiment, the bicyclic peptides of the invention find utility in and may be used for the treatment of vaso-occlusive crisis. In a further embodiment, the bicyclic peptides may be used in the treatment or prevention of metastasis. In a yet further embodiment, the bicyclic peptides may be used in the treatment or prevention of cardiac inflammation, fibrosis and atherosclerotic plaques.

It will be appreciated that multimeric binding complexes as defined herein which comprise at least one peptide ligand which binds to a cell adhesion molecule, such as P-selectin, on endothelial cells will also be useful in the treatment of cancer and non-cancer diseases and disorders, such as vaso-occlusive crisis, cardiac inflammation, fibrosis and atherosclerotic plaques.

It will also be appreciated that multimeric binding complexes as defined herein which comprise at least one peptide ligand which binds to a cell adhesion molecule, such as P-selectin, on endothelial cells will also be useful in the treatment of COVID-19.

COVID-19 is primarily a respiratory condition, it has also been viewed as a multi-system disease. Abundant evidence reveals that the SARS-CoV-2 virus can attack organs and tissues that express angiotensin-converting enzyme 2 (ACE2) receptor, which include heart, renal, liver, pancreas, gastrointestinal tract, brain, and endothelial cells, subsequently trigger inflammatory cascades and blood clotting. COVID-19 may cause severe vascular complications, which consequently lead to endothelial dysfunction, overproduction cytokines, and pathological cellular interactions. Red blood cells (RBCs) are the most abundant cellular components of blood and responsible for the transportation of oxygen, removal of waste, and delivery of nutrients throughout the human body. Prior studies suggested that SARS-CoV-2 infection can also damage RBC membrane, decrease hemoglobin (Hb) concentration, and prevent red blood cell (RBC) formation, resulting in serious anemia and irreversible organ damage. Thus, one plausible pathogenic mechanism of the “post-COVID syndrome” is the combination of red blood cell abnormality and build-up of inflammatory factors, which in turn cause inadequate oxygen delivery, hypercoagulation, and vascular complications, and contribute, at least in part, to the prolonged symptoms observed in a significant portion of COVID patients, including chronic fatigue, excise intolerance, joint and chest pain, and brain fog. Since dysregulation of P-selectin is a key factor of COVID-19 pathology, inhibition of inflammation-driven, P-selectin mediated blood cell adhesion to endothelium could be a

promising therapeutic strategy to mitigate adverse symptoms, facilitate function recovery, and improve long-term health of millions of individuals affected by this disease.

References herein to the term "prevention" involves administration of the protective composition prior to the induction of the disease. "Suppression" refers to administration of the composition after an inductive event, but prior to the clinical appearance of the disease. "Treatment" involves administration of the protective composition after disease symptoms become manifest.

Animal model systems which can be used to screen the effectiveness of the peptide ligands in protecting against or treating the disease are available. The use of animal model systems is facilitated by the present invention, which allows the development of polypeptide ligands which can cross react with human and animal targets, to allow the use of animal models.

The invention is further described below with reference to the following examples.

EXAMPLES

Materials and Methods

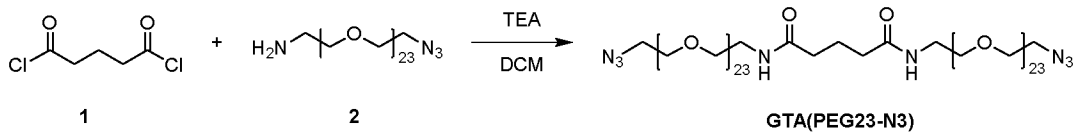
Preparation of Monomeric Bicyclic Peptide Ligands (General Method)

Bicycle peptides were synthesized on Rink amide resin using standard Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase peptide synthesis, either by manual coupling (for large scale) or using a Biotage SyroII automated peptide synthesizer (for small scale). Following TFA-based cleavage from the resin, peptides were precipitated with diethyl ether and dissolved in 50:50 acetonitrile/water. The crude peptides (at ~1 mM concentration) were then cyclized with 1.3 equiv. of the scaffold, using ammonium bicarbonate (100 mM) as a base. Completion of cyclization was determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) or LC−MS. Once complete, the cyclization reaction was quenched using N-acetyl cysteine (10 equiv. with respect to the peptide), and the solutions were lyophilized. The residue was dissolved in an appropriate solvent and purified by RP-HPLC. Peptide fractions of sufficient purity and the correct molecular weight (verified by either MALDI-TOF and HPLC or LC−MS) were pooled and lyophilized. Concentrations were determined by UV absorption using the extinction coefficient at 280 nm, which was based on Trp/Tyr content.

All amino acids, unless noted otherwise, were used in the L-configurations.

Preparation of Dimeric/Tandem Binding Complexes (Exemplified with BCY5455)

Synthesis of Hinge GTA(PEG23-N3):



1 2 GTA(PEG23-N3)

To a solution of compound **1** (10 mg, 59.17 μmol, 7.58 μL, 1 eq.) in DCM (2 mL) was added Triethylamine (17.96 mg, 177.51 μmol, 24.71 μL, 3 eq.). Compound **2** (132.0 mg, 120.08 μmol, 2.03 eq.) was then added. The mixture was stirred at 30°C for 2 hr. LC-MS showed compound **1** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by preparative HPLC. **GTA(PEG23-N3)** (82 mg, 35.02 μmol, 59.19% yield, 98% purity) was obtained as a white solid. (MW: 2294.69. Observed *m/z*: 1156.31 $[M+H_3O+H]^{2+}$).

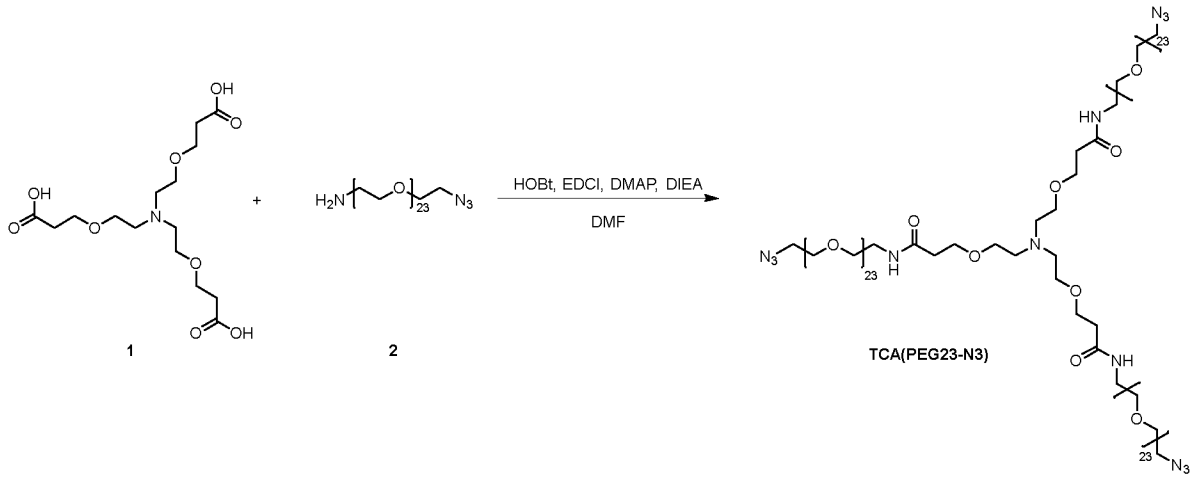
Preparation of BCY5455:

GTA(PEG23-N3)
3

To a solution of **GTA(PEG23-N3)** (10 mg, 4.36 μmol, 1 eq.), **BCY10910** (20.00 mg, 9.72 μmol, 2.23 eq.) and THPTA (0.4 M, 10.89 μL, 1 eq.) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 3 times), and then CuSO4 (0.4 M, 10.89 μL, 1 eq.) and VcNa (0.4 M, 21.79 μL, 2 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40°C for 12 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z was detected. The residue was purified by preparative HPLC. **BCY5455** (13.6 mg, 2.07 μmol, 47.56% yield, 97.70% purity) was obtained as a white solid. (MW: 6411.26 observed *m/z*: 1603.1 $[M+4H]^{4+}$, 1282.4 $[M+5H]^{5+}$, 1068.95 $[M+6H]^{6+}$).

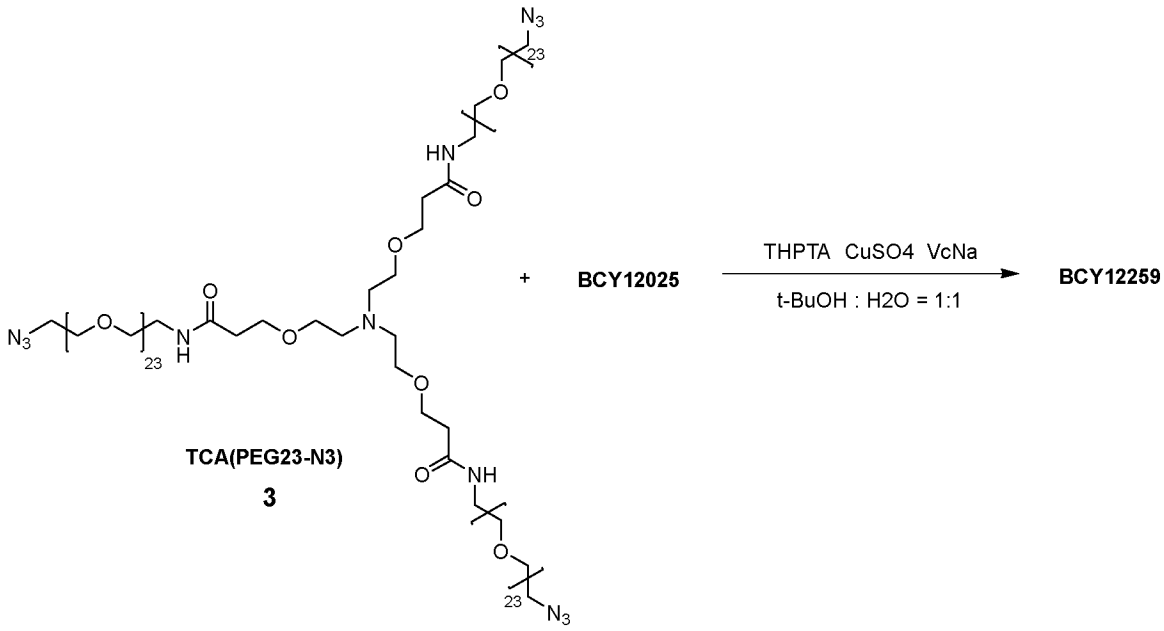
Preparation of Trimeric Binding Complexes (Exemplified with BCY12259)

Synthesis of Hinge TCA(PEG23-N3):



To a solution of compound **1** (32 mg, 79.63 μmol, 1.0 eq., HCl) and compound **2** (264.00 mg, 240.15 μmol, 3.02 eq.) in DMF (2 mL) was added HOBt (35.84 mg, 265.24 μmol, 3.33 eq.), EDCI (51.20 mg, 267.08 μmol, 3.35 eq.), DMAP (29.19 mg, 238.90 μmol, 3.0 eq.) and DIPEA (61.75 mg, 477.81 μmol, 83.23 μL, 6.0 eq.). The mixture was stirred at 25-30°C for 12 hr. LC-MS showed compound **1** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by preparative HPLC. **TCA(PEG23-N3)** (214 mg, 53.36 μmol, 67.01% yield, 90% purity) was obtained as a light yellow oil. (MW: 3609.23 observed *m/z*: 1214 $[M+2H_3O+H]^{3+}$).

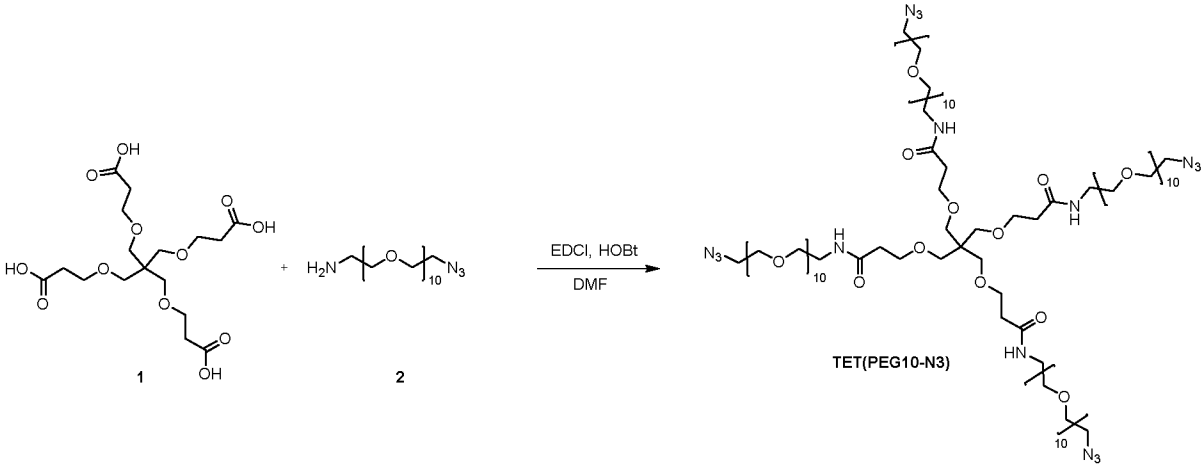
Preparation of BCY12259:



To a solution of **TCA(PEG23-N3)** (10 mg, 2.77 μmol, 1 eq.), **BCY12025** (17 mg, 8.41 μmol, 3.03 eq.) and THPTA (0.4 M, 6.93 μL, 1 eq.) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 3 times), and then CuSO4 (0.4 M, 6.93 μL, 1 eq.) and VcNa (0.4 M, 13.85 μL, 2 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40°C for 12 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z was detected. The residue was purified by preparative HPLC. **BCY12259** (14 mg, 1.34 μmol, 48.28% yield, 92.46% purity) was obtained as a white solid. (MW: 9676.10 observed *m/z*: 1382.2 $[M+7H]^{7+}$, 1209.3 $[M+8H]^{8+}$, 1075.1 $[M+9H]^{9+}$, 967.3 $[M+10H]^{10+}$).

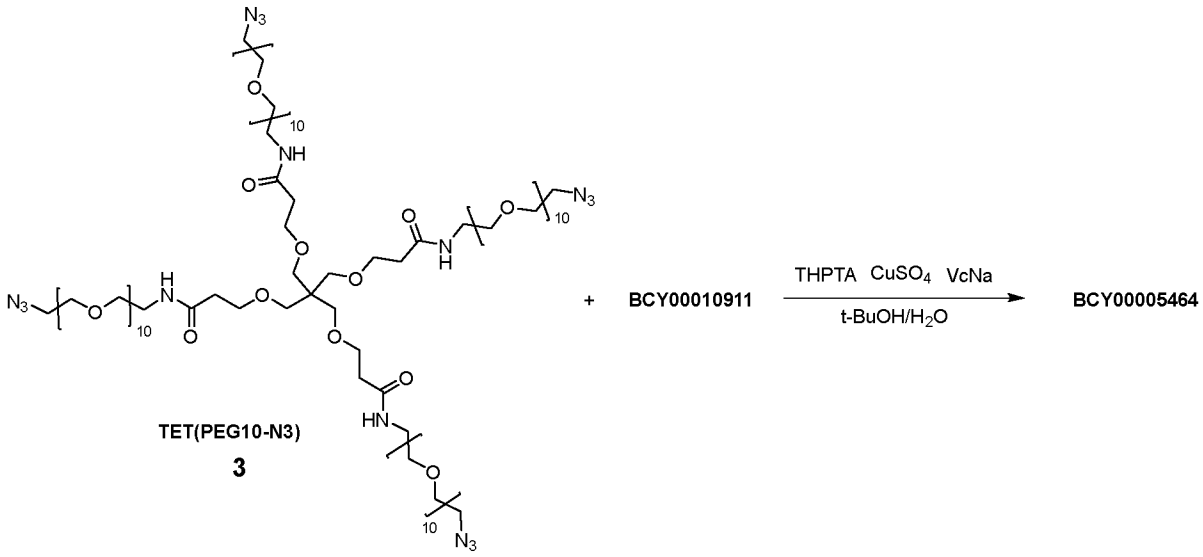
Preparation of Tetrameric Binding Complexes (Exemplified with BCY5464)

Synthesis of Hinge TET(PEG10-N3):



A mixture of compound **1** (52.0 mg, 122.53 μmol, 1 eq.), compound **2** (273.0 mg, 518.40 μmol, 4.23 eq.), EDCI (117.5 mg, 612.64 μmol, 5.0 eq.), and HOBT (72.8 mg, 539.12 μmol, 4.08 eq.) was dissolved in DMF (2 mL), and then DIPEA (126.7 mg, 980.22 μmol, 170.74 μL, 8.0 eq.) was added and the solution mixed well. The mixture was stirred at 25°C for 12 hr. LC-MS showed one main peak with desired m/z. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by preparative HPLC. **TET(PEG10-N3)** (147.8 mg, 55.79 μmol, 45.53% yield, 92.81% purity) was obtained as a white solid. (MW: 2458.81. observed *m/z*: 810.7 $[M-28+3H]^{3+}$).

Preparation of BCY5464:



A solution of **TET(PEG10-N3)** (5 mg, 2.03 µmol, 1 eq.), **BCY10911** (19 mg, 8.81 µmol, 4.33 eq.) and THPTA (0.4 M, 5.0 µL, 1 eq.) in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2) was prepared. CuSO4 (0.4 M, 5.0 µL, 1 eq.) and VcNa (0.4 M, 10.0 µL, 2 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40°C for 16 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z was detected. The crude reaction was combined and purified by preparative HPLC. **BCY5464** (12.6 mg, 92.6% purity) was obtained as a white solid. (MW: 11088.49 observed *m/z*: 1233.6 $[M+9H]^{9+}$, 1387.2 $[M+8H]^{8+}$).

It will be appreciated that, in general, all multimeric binding complexes of the invention may be prepared in accordance with these methods using the appropriate bicyclic peptide ligand(s).

ANALYTICAL DATA

The following multimeric binding complexes of the invention were analysed using mass spectrometry and HPLC and the data are shown in Tables 4 and 5 below.

HPLC setup was as follows:

Methods A, B, and E:

Mobile Phase: A: 0.1%TFA in H_2O B: 0.1%TFA in ACN

Flow: 1.0 ml/min

Column: Gemini-NX C18 5 µm 110A 150*4.6mm

Instrument: Agilent 1200 HPLC-BE(1-614)

Gradients:

Method	Gradient Description
A	25-55% B over 20 minutes
B	20-50% B over 20 minutes
E	30-60% B over 20 minutes

Method C:

Mobile Phase: A: 0.1%TFA in H_2O B: 0.1%TFA in ACN

Flow: 0.4 ml/min

Column: Kintex 1.7 µm C18 100A 2.1mm*150mm

Instrument: Agilent UPLC 1290

Column temperature: 30°C

Gradient:

Time (min)	% A	% B
0	75	25
10	45	55
10.01	10	90
12	10	90

Method D:

Mobile phase: 0.1% TFA AcN/Water

Flow: 1.00 ml/min

Column: KINETEXPSC18

Instrument: Aglient LC2

Gradient: 5-95% B in 15.5 minutes

Table 4: HPLC and Mass Spectrometry Data for Monomeric Bicycle Peptide Ligands of the Invention

Monomer ID	Molecular Weight	Mass Spectrometry - Observed m/z	HPLC Retention Time (min)	Method
BCY12027	1825.05	912.6 $[M+2H]^{2+}$	9.82	B
BCY11648	1814.03	907.58 $[M+2H]^{2+}$	8.42	B
BCY11279	2031.25	1016.64 $[M+2H]^{2+}$	6.30	D
BCY11281	2034.26	1018.03 $[M+2H]^{2+}$	6.20	D

BCY09717	1878.08	939.75 $[M+2H]^{2+}$	5.95	D
BCY10194	1978.21	989.4 $[M+2H]^{2+}$	10.08	B
BCY10910	2058.29	1029.14 $[M+2H]^{2+}$	14.03	B
BCY10911	2157.42	1079.65 $[M+2H]^{2+}$	10.31	A
BCY12025	2022.28	1011.16 $[M+2H]^{2+}$	9.58	B
BCY12026	2033.31	1016.64 $[M+2H]^{2+}$	10.91	B
BCY11890	2197.5	732.9 $[M+3H]^{3+}$, 1099.4 $[M+2H]^{2+}$	9.47	A
BCY18041	2186.5	729.5 $[M+3H]^{3+}$, 1093.9 $[M+2H]^{2+}$	11.10	B

Table 5: HPLC and Mass Spectrometry Data for Multimeric Binding Complexes of the Invention

Complex ID	Molecular Weight	Mass Spectrometry - Observed m/z	HPLC Retention Time (min)	Method
BCY5454	5265.91	1754.7 $[M+3H]^{3+}$, 1316.4 $[M+4H]^{4+}$, 1053.6 $[M+5H]^{5+}$	12.32	A
BCY5455	6411.28	1603.1 $[M+4H]^{4+}$, 1282.4 $[M+5H]^{5+}$, 1068.95 $[M+6H]^{6+}$	13.06	A
BCY5456	5464.18	1822.4 $[M+3H]^{3+}$, 1367.2 $[M+4H]^{4+}$, 1093.5 $[M+5H]^{5+}$	5.89	C
BCY5457	6609.54	1322.3 $[M+5H]^{5+}$, 1101.9 $[M+6H]^{6+}$, 944.65 $[M+7H]^{7+}$	12.78	A
BCY12257	6339.26	1584.6 $[M+4H]^{4+}$, 1267.7 $[M+5H]^{5+}$, 1056.5 $[M+6H]^{6+}$, 905.6 $[M+7H]^{7+}$	9.04	A
BCY12258	6361.31	1273.1 $[M+5H]^{5+}$, 1063.7 $[M+6H]^{6+}$, 909.5 $[M+7H]^{7+}$	13.43	B
BCY5458	8066.07	1345.0 $[M+6H]^{6+}$, 1153.14 $[M+7H]^{7+}$	12.63	A
BCY5459	9784.12	1223.3 $[M+8H]^{8+}$, 1087.6 $[M+9H]^{9+}$	13.32	A
BCY5460	8363.47	1674.2 $[M+5H]^{5+}$, 1394.4 $[M+6H]^{6+}$	12.73	A
BCY5461	10081.52	1262.83 $[M+8H]^{8+}$, 1124.36 $[M+9H]^{9+}$	13.25	A
BCY12259	9676.1	1382.2 $[M+7H]^{7+}$, 1209.3 $[M+8H]^{8+}$, 1075.1 $[M+9H]^{9+}$, 967.3 $[M+10H]^{10+}$	9.03	A
BCY12260	9709.16	1386.5 $[M+7H]^{7+}$, 1213.3 $[M+8H]^{8+}$, 1078.4 $[M+9H]^{9+}$, 970.9 $[M+10H]^{10+}$	10.03	A

BCY5462	10691.99	1188.41 $[M+9H]^{9+}$	14.14	A
BCY5463	12996.75	1855.2 $[M+7H]^{7+}$, 1299.4 $[M+10H]^{10+}$	14.76	A
BCY5464	11088.51	1585.6 $[M+7H]^{7+}$	14.32	A
BCY5465	13379.35	1673.4 $[M+8H]^{8+}$	14.56	A
BCY12261	12838.69	1427.4 $[M+9H]^{9+}$, 1286.6 $[M+10H]^{10+}$, 1169.6 $[M+11H]^{11+}$	9.91	A
BCY12262	12882.78	1611.3 $[M+8H]^{8+}$, 1432.3 $[M+9H]^{9+}$, 1290.6 $[M+10H]^{10+}$, 1173.5 $[M+11H]^{11+}$	10.95	A
BCY11903	13539	13557 (TOF deconvoluted mass)	12.58	A
BCY19238	13495	13495 (TOF deconvoluted mass)	11.14	A
BCY19242	10202	10202 (TOF deconvoluted mass)	8.78	E
BCY19239	10169	10169 (TOF deconvoluted mass)	13.73	B
BCY19243	6689.7	6689 (TOF deconvoluted mass)	11.44	A
BCY19240	6667.6	6668 (TOF deconvoluted mass)	13.63	B

BIOLOGICAL DATA

1. SPR Binding Assay

Biacore experiments were performed to determine ka (M-1s-1), kd (s-1), KD (nM) values of monomeric peptides binding to human Selectin proteins. Recombinant biotinylated human P-Selectin protein was used. For analysis of binding, either a Biacore 3000 or S200 instrument was used with a CM5 sensor chip (GE Healthcare). For sensor chip surface preparation, streptavidin was immobilized on the chip using standard amine-coupling chemistry at 25°C with HBS-N (10 mM HEPES, 0.15 M NaCl, pH 7.4) as the running buffer. The carboxymethyl dextran surface was activated with a 12 min injection of a 1:1 ratio of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/0.1 M N-hydroxy succinimide (NHS) at a flow rate of 10 µl/min. For capture of streptavidin, protein was diluted to 0.2 mg/ml in 10 mM sodium acetate (pH 4.5) and captured by injecting 70 µl onto the activated chip surface. Residual activated groups were blocked with a 7 min injection of 1 M ethanolamine (pH 8.5). Biotinylated protein stock was diluted 1:100 in HBS-N and captured on one flow cell at 5 µl/min to a level of 1000-1500 RU. Buffer was changed to PBS/0.05% Tween 20 and a dilution series of the peptides was prepared in this buffer with a final DMSO concentration of 0.5%. The top concentration of the analyte varied from 200 nM to 50 µM; depending on predicted affinity of the interaction. In all cases a titration was performed with either seven 2-fold dilutions or five 3-fold dilutions. The SPR analysis was run at 25°C with a flow rate of 50-80 µl/min with 60

seconds association and a suitable disassociation period (100-900 seconds). Data were corrected for DMSO excluded volume effects. All data were double-referenced for blank injections and reference surface using standard processing procedures and data processing and kinetic fitting were performed using Scrubber software, version 2.0c (BioLogic Software). Data were fitted using mass transport model allowing for mass transport effects where appropriate.

Table 6: Affinity K_D Values Determined by SPR

Peptide Number	***K_D (nM)***
BCY12027	5.36
BCY11648	128
BCY11279	690
BCY11281	2020
BCY9717	7600
BCY10194	9500
BCY5454	400
BCY5455	1250
BCY5456	500
BCY5457	969
BCY5458	400
BCY5459	169
BCY5460	239
BCY5461	230
BCY5462	88.0
BCY5463	53.0
BCY5464	37.3
BCY5465	140
BCY12257	11.6
BCY12258	0.409
BCY12259	0.233
BCY12260	0.109
BCY12261	0.019
BCY12262	0.017

2. Receptor Ligand Inhibition Assay

Bicycle test samples, SelG1 (Crizanlizumab) were serially diluted maintaining 0.5% DMSO in assay buffer (25mM HEPES, 121mM NaCl, 5.4mM KCl, 0.8mM $MgCl_2$, 1.8mM $CaCl_2$, 6mM $NaHCO_2$, 5.5mM glucose, 1% BSA, pH7.4) and transferred to 384 well clear bottom plates (Corning, 3712) with a final volume of 6µL. 6µL of P-Selectin in imaging buffer (25mM HEPES, 121mM NaCl, 5.4mM KCl, 0.8mM $MgCl_2$, 1.8mM $CaCl_2$, 6mM $NaHCO_2$, 5.5mM glucose, pH7.4) was added to give a 5nM final concentration and incubated at room temperature for 15 minutes. HL-60 cells (ATCC, CCL-240) mixed with 1µg/mL far red labelled wheat germ agglutinin (WGA, ThermoFisher W21404) and 1µg/mL AlexaFluor 488- streptavidin (Invitrogen S11223) were added to the plate and incubated for 2 hours at room temperature in the absence of light. Staining and antibody binding were quantified by simultaneously scanning the microplates with 488 nm and 640 nm lasers on a mirrorball microplate cytometer, AF488 on FL2 triggering on AF647-WGA on FL4. Data was analysed in Dotmatics, using a four parameter logistic fit to generate IC50 values and % response. Percent inhibition was calculated using 100 - % response at top concentration. Selected peptides were tested in this assay and the results (IC_{50} and % inhibition data) are shown in Table 7 where data displayed is the geomean of n=3.

Table 7: Effect of P-Selectin binding Bicycles on the P-Selectin:PSGL1 interaction

BCY number	IC_{50} (nM)	Inhibition at top concentration (%)
SelG1	4.75	96
BCY9717	>10875	20
BCY11279	>3539	24
BCY11648	>117	37
BCY12027	>36.8	36
BCY12257	>78.4	54
BCY12258	>97.7	57
BCY12259	14.4	61
BCY12260	9.62	64
BCY12261	10.4	68
BCY12262	3.29	68

3. Cell Adhesion Bioassay Characterization of BCY12262

Background

In flow adhesion assays (Figure 1) microfluidic channels are coated with proteins that mimic the endothelium, including specific binding receptors of interest (such as P-Selectin). Then a sample of cells and test articles is flown at physiologically relevant flow conditions through the channel and the number of cells that adhere to the channel surface is quantified. P-selectin is a cell adhesion molecule on the surfaces of activated endothelial cells. White Blood Cells (WBC) materially contribute to adhesion on P-selectin in Whole Blood. White Blood Cells are isolated from the blood sample and the concentration normalized to 5 million/mL.

Methodology

A commercial well plate microfluidic flow adhesion system, BioFlux 1000Z (Fluxion Bio, San Francisco, CA, USA) was utilized. Isolated WBC suspension from a single convalescent COVID-19 patient (at 5 million cells/ml in Hank's Balanced Salt Solution buffer) was perfused through P-selectin (5 µg/ml)-coated microfluidic channels (350 µm wide x 70 µm tall) using pulsatile (1·67 Hz) shear stress (1 dyne/cm^2) and washed with the buffer at the same flow rate to eliminate non-adhering cells. Images were acquired with a high-resolution CCD camera and analyzed with Montage imaging software (Molecular Devices, Downingtown, PA, USA). An adhesion index (AI) was established for each sample by quantifying adherent cells within a standard viewing area (cells/mm^2).

Results

The results of this analysis are shown in Figure 2 where it can be seen that significant inhibition of white blood cell adhesion was observed for BCY12262 that compares well to the positive control antibody (crizanlizumab). BCY12262 showed a strong dose-response relationship with approximately 70 percent inhibition reached at higher doses (100 nM to 10µM). Negative control peptide BCY17800 that does not bind to P-selectin showed no inhibition in the flow adhesion assay at 1µM.

CLAIMS

1. A peptide ligand specific for P-selectin comprising a polypeptide comprising at least three reactive groups, separated by at least two loop sequences, and a molecular scaffold which forms covalent bonds with the reactive groups of the polypeptide such that at least two polypeptide loops are formed on the molecular scaffold.

2. The peptide ligand as defined in claim 1, wherein said reactive groups comprise cysteine residues.

3. The peptide ligand as defined in claim 1 or claim 2, wherein said peptide ligand comprises the motif WCDV, or a modified derivative thereof.

4. The peptide ligand as defined in any one of claims 1 to 3, wherein said loop sequences comprise 4 or 6 amino acids.

5. The peptide ligand as defined in any one of claims 1 to 4, wherein said loop sequences comprise three cysteine residues separated by two loop sequences the first of which consists of 4 amino acids and the second of which consists of 6 amino acids.

6. The peptide ligand as defined in claim 5, wherein said peptide ligand comprises an amino acid sequence of:

C_i-X_1-X_2-X_3-X_4-C_{ii}-D-V-X_5-X_6-X_7-X_8-C_{iii} (SEQ ID NO: 1),

wherein

X_1 represents D or Y;

X_2 represents A or M;

X_3 represents D or E;

X_4 represents W, 1Nal or Trp(Me);

X_5 represents P or T;

X_6 represents S or D;

X_7 represents L or Y;

X_8 represents P or G;

wherein 1Nal represents 1-naphthylalanine, Trp(Me) represents methyl-tryptophan and C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, or a modified derivative, or a pharmaceutically acceptable salt thereof.

7. The peptide ligand as defined in claim 6, wherein X_4 represents W.

8. The peptide ligand as defined in claim 6 or claim 7, wherein the peptide ligand of C_i-X_1-X_2-X_3-X_4-C_{ii}-D-V-X_5-X_6-X_7-X_8-C_{iii} (SEQ ID NO: 1) comprises an amino acid sequence selected from:

C_iDAD[1Nal]C_{ii}DVPSLPC_{iii} (SEQ ID NO: 2);

C_iDADWC_{ii}DVPSLPC_{iii} (SEQ ID NO: 3);

C_iYME[1Nal]C_{ii}DVTDYGC_{iii} (SEQ ID NO: 4);

C_iYME[Trp(Me)]C_{ii}DVTDYGC_{iii} (SEQ ID NO: 5); and

C_iYMEWC_{ii}DVTDYGC_{iii} (SEQ ID NO: 6);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, or a modified derivative, or a pharmaceutically acceptable salt thereof), such as:

A-(SEQ ID NO: 2)-A (herein referred to as BCY12027);

H_2N-A-(SEQ ID NO: 2)-A-[K(PYA)] (herein referred to as BCY12026);

A-(SEQ ID NO: 3)-A (herein referred to as BCY11648);

H_2N-A-(SEQ ID NO: 3)-A-[K(PYA)] (herein referred to as BCY12025);

Ac-A-(SEQ ID NO: 4)-A (herein referred to as BCY11279);

A-(SEQ ID NO: 4)-A-[K(PYA)] (herein referred to as BCY11890);

Ac-A-(SEQ ID NO: 5)-A (herein referred to as BCY11281);

Ac-(SEQ ID NO: 6) (herein referred to as BCY9717);

A-(SEQ ID NO: 6)-A (herein referred to as BCY10194);

A-(SEQ ID NO: 6)-A-[K(PYA)] (herein referred to as BCY18041);

[PYA]-A-(SEQ ID NO: 6)-A-NH_2 (herein referred to as BCY10910); and

Ac-A-(SEQ ID NO: 6)-[K(PYA)]-NH_2 (herein referred to as BCY10911),

wherein PYA represents propargyl-acid, in particular:

A-(SEQ ID NO: 2)-A (herein referred to as BCY12027);

H_2N-A-(SEQ ID NO: 2)-A-[K(PYA)] (herein referred to as BCY12026);

H_2N-A-(SEQ ID NO: 3)-A-[K(PYA)] (herein referred to as BCY12025); and

A-(SEQ ID NO: 3)-A (herein referred to as BCY11648); more particularly:

A-(SEQ ID NO: 2)-A (herein referred to as BCY12027); and

H_2N-A-(SEQ ID NO: 2)-A-[K(PYA)] (herein referred to as BCY12026); most particularly;

A-(SEQ ID NO: 2)-A (herein referred to as BCY12027).

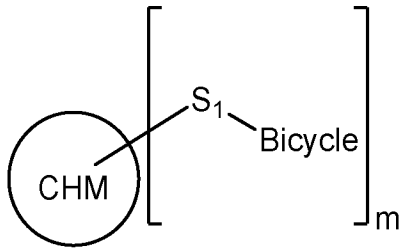
9. The peptide ligand as defined in any one of claims 1 to 8, wherein the molecular scaffold is selected from: 1,1',1''-(1,3,5-triazinane-1,3,5-triyl)triprop-2-en-1-one (TATA) and 1,1',1''-(1,4,7-triazonane-1,4,7-triyl)tris(2-chloroethan-1-one) (TCAZ) or the bromo derivative 1,1',1''-(1,4,7-triazonane-1,4,7-triyl)tris(2-bromothan-1-one) (TBAZ).

10. The peptide ligand as defined in any one of claims 1 to 9, wherein the pharmaceutically acceptable salt is selected from the free acid or the sodium, potassium, calcium or ammonium salt.

11. A multimeric binding complex which comprises at least two peptide ligands, wherein at least one peptide ligand is specific for P-selectin as defined in any one of claims 1 to 10 and said peptide ligands may be the same or different, each of which comprises a polypeptide comprising at least three reactive groups, separated by at least two loop sequences, and a molecular scaffold which forms covalent bonds with the reactive groups of the polypeptide such that at least two polypeptide loops are formed on the molecular scaffold.

12. The multimeric binding complex as defined in claim 11, wherein each peptide ligand is connected to a central hinge moiety by a spacer group.

13. The multimeric binding complex as defined in claim 11 or claim 12, which comprises a compound of formula (I):



(I)

wherein CHM represents a central hinge moiety;
S_1 represents a spacer group;
Bicycle represents a peptide ligand as defined in any one of claims 1 to 10; and
m represents an integer selected from 2 to 10.

14. The multimeric binding complex as defined in any one of claims 11 to 13, wherein said peptide ligands are specific for the same target, in particular P-selectin.

15. The multimeric binding complex as defined in any one of claims 11 to 14, wherein the multimeric binding complex comprises at least two identical peptide ligands.

16. The multimeric binding complex as defined in claim 15, wherein the multimeric binding complex comprises two identical peptide ligands and is selected from: BCY5454, BCY5455,

BCY5456, BCY5457, BCY12257, BCY12258, BCY19243, and BCY19240, such as BCY5454, BCY5455, BCY5456, BCY5457, BCY12257, and BCY12258.

17. The multimeric binding complex as defined in claim 15, wherein the multimeric binding complex comprises three identical peptide ligands and is selected from: BCY5458, BCY5459, BCY5460, BCY5461, BCY12259, BCY12260, BCY19242, and BCY19239, such as BCY5458, BCY5459, BCY5460, BCY5461, BCY12259, and BCY12260.

18. The multimeric binding complex as defined in claim 15, wherein the multimeric binding complex comprises four identical peptide ligands and is selected from: BCY5462, BCY5463, BCY5464, BCY5465, BCY12261, BCY12262, BCY11903, and BCY19238, such as BCY5462, BCY5463, BCY5464, BCY5465, BCY12261, and BCY12262.

19. The multimeric binding complex as defined in any one of claims 11 to 18, wherein the multimeric binding complex comprises at least two differing peptide ligands.

20. A drug conjugate comprising the peptide ligand as defined in any one of claims 1 to 10 or the multimeric binding complex as defined in any one of claims 11 to 19, conjugated to one or more effector and/or functional groups.

21. A pharmaceutical composition which comprises the peptide ligand as defined in any one of claims 1 to 10, the multimeric binding complex as defined in any one of claims 11 to 19 or the drug conjugate of claim 20, in combination with one or more pharmaceutically acceptable excipients.

22. The peptide ligand as defined in any one of claims 1 to 10, the multimeric binding complex as defined in any one of claims 11 to 19, or the drug conjugate of claim 20, for use in preventing, suppressing or treating a disease or disorder mediated by cell adhesion molecules.

23. The peptide ligand, multimeric binding complex or drug conjugate for use as defined in claim 22, wherein the cell adhesion molecule is P-selectin.

24. The peptide ligand, multimeric binding complex or drug conjugate for use as defined in claim 22 or claim 23, wherein the disease or disorder is vaso-occlusive crisis.

25. The peptide ligand, multimeric binding complex or drug conjugate for use as defined in any one of claims 22 to 24, wherein the disease or disorder is related to sickle cell disease or sickle cell anaemia.

26. The peptide ligand as defined in any one of claims 1 to 10, the multimeric binding complex as defined in any one of claims 11 to 19, or the drug conjugate of claim 20, for use in preventing, suppressing or treating cancer.

27. The peptide ligand, multimeric binding complex or drug conjugate for use as defined in claim 26, wherein the prevention, suppression or treatment of cancer comprises the blocking, prevention or suppression of cancer or cancer cell metastasis.

28. The peptide ligand, multimeric binding complex or drug conjugate for use as defined in any one of claims 22 to 24, wherein the disease or disorder is COVID-19.

1/1

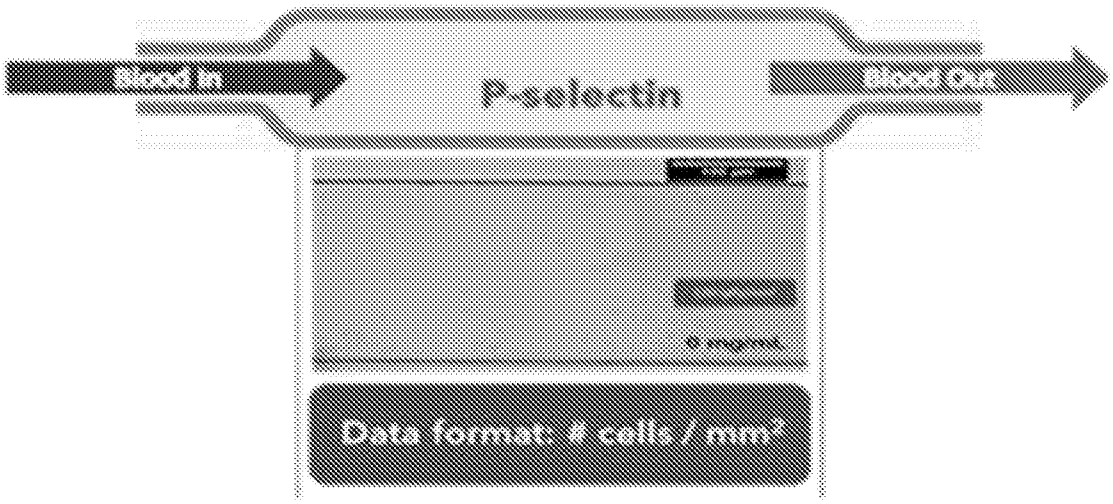


FIGURE 1

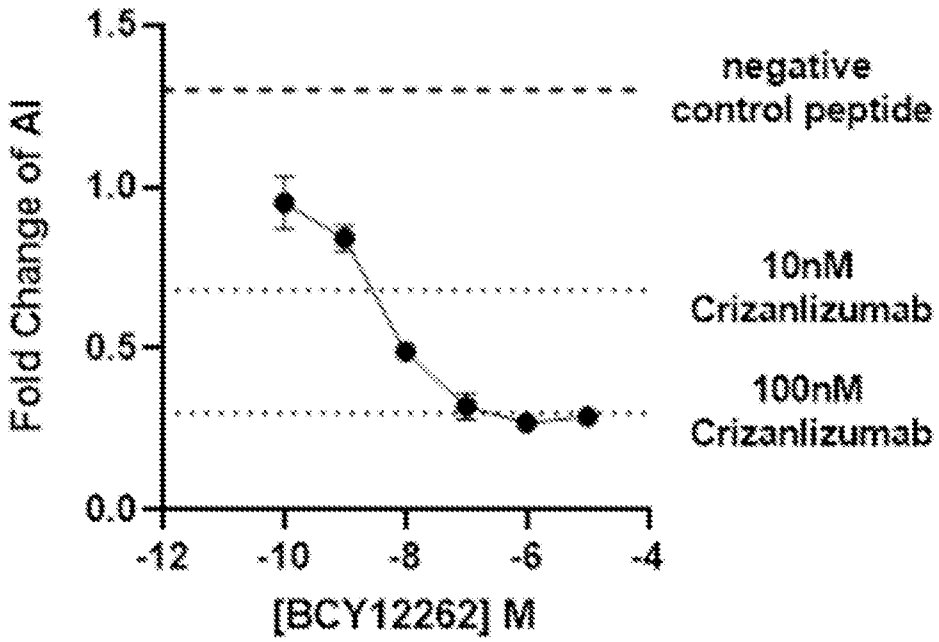


FIGURE 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2022/050995

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K7/08 A61K47/64 A61P35/00
ADD.

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)
C07K A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAYCIE DEYLE ET AL: "Phage Selection of Cyclic Peptides for Application in Research and Drug Development", ACCOUNTS OF CHEMICAL RESEARCH, vol. 50, no. 8, 18 July 2017 (2017-07-18), pages 1866-1874, XP055562233, US ISSN: 0001-4842, DOI: 10.1021/acs.accounts.7b00184 the whole document ----- -/--	1-28

[x] Further documents are listed in the continuation of Box C.

[x] 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
25 August 2022	02/09/2022

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cervigni, S

1

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2022/050995

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KALE SANGRAM S ET AL: "Cyclization of peptides with two chemical bridges affords large scaffold diversities", NATURE CHEMISTRY, NATURE PUBLISHING GROUP UK, LONDON, vol. 10, no. 7, 30 April 2018 (2018-04-30) , pages 715-723, XP036530421, ISSN: 1755-4330, DOI: 10.1038/S41557-018-0042-7 [retrieved on 2018-04-30] the whole document	1-28
X	WO 2018/197509 A1 (BICYCLERD LTD [GB]) 1 November 2018 (2018-11-01) the whole document	1-28
X	WO 2020/084305 A1 (BICYCLETX LTD [GB]) 30 April 2020 (2020-04-30) the whole document	1-28
X	WO 2018/197893 A1 (BICYCLETX LTD [GB]) 1 November 2018 (2018-11-01) the whole document	1-28
X	WO 2019/162682 A1 (BICYCLETX LTD [GB]) 29 August 2019 (2019-08-29) the whole document	1-28
X	WO 03/020753 A1 (YAMANOUCHI EUROP BV [NL]; MOLENAAR THOMAS JACOBUS MARIA [NL] ET AL.) 13 March 2003 (2003-03-13) the whole document page 23; claim 1	1-28
X	MOLENAAR T J M ET AL: "Specific inhibition of P-selectin-mediated cell adhesion by phage display-derived peptide antagonists", BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 100, no. 10, 15 November 2002 (2002-11-15), pages 3570-3577, XP002223456, ISSN: 0006-4971, DOI: 10.1182/BLOOD-2002-02-0641 the whole document table 1	1-28
X	WO 2004/105783 A1 (YAMANOUCHI PHARMA CO LTD [JP] ET AL.) 9 December 2004 (2004-12-09) the whole document	1-28

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2022/050995

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. [X] forming part of the international application as filed:

      [X] 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 13ter.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 13ter.1(a)).

      [ ] on paper or in the form of an image file (Rule 13ter.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:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2022/050995

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2018197509 A1	01-11-2018	EP 3615942 A1	04-03-2020
		US 2020407709 A1	31-12-2020
		WO 2018197509 A1	01-11-2018
WO 2020084305 A1	30-04-2020	CN 112955459 A	11-06-2021
		EP 3870597 A1	01-09-2021
		JP 2022512779 A	07-02-2022
		US 2020131228 A1	30-04-2020
		US 2021147484 A1	20-05-2021
		WO 2020084305 A1	30-04-2020
WO 2018197893 A1	01-11-2018	EP 3615550 A1	04-03-2020
		US 2018311300 A1	01-11-2018
		US 2021046145 A1	18-02-2021
		WO 2018197893 A1	01-11-2018
WO 2019162682 A1	29-08-2019	AU 2019224659 A1	15-10-2020
		BR 112020014576 A2	08-12-2020
		CA 3091775 A1	29-08-2019
		CN 111902429 A	06-11-2020
		EP 3755725 A1	30-12-2020
		IL 276803 A	29-10-2020
		JP 2021514953 A	17-06-2021
		KR 20200128518 A	13-11-2020
		SG 11202007678Q A	29-09-2020
		US 2019263866 A1	29-08-2019
		US 2021101933 A1	08-04-2021
		WO 2019162682 A1	29-08-2019
WO 03020753 A1	13-03-2003	AT 446311 T	15-11-2009
		BR 0212219 A	21-09-2004
		CA 2459230 A1	13-03-2003
		CN 1549824 A	24-11-2004
		EP 1288222 A1	05-03-2003
		EP 1423413 A1	02-06-2004
		ES 2337663 T3	28-04-2010
		HR P20040127 A2	30-06-2004
		HU 0401728 A2	29-11-2004
		JP 4227520 B2	18-02-2009
		JP 2005501544 A	20-01-2005
		MX PA04002055 A	08-07-2004
		US 2005004035 A1	06-01-2005
		WO 03020753 A1	13-03-2003
		ZA 200400956 B	18-04-2005
WO 2004105783 A1	09-12-2004	AT 341337 T	15-10-2006
		CA 2527438 A1	09-12-2004
		DE 602004002699 T2	16-08-2007
		EP 1481683 A1	01-12-2004
		EP 1638588 A1	29-03-2006
		ES 2273260 T3	01-05-2007
		JP 4771291 B2	14-09-2011
		JP 2007500200 A	11-01-2007
		US 2007185348 A1	09-08-2007
		WO 2004105783 A1	09-12-2004