Abstract:
The present invention relates to labelled cMet binding peptides suitable for optical imaging in vivo. The peptides are labelled with a benzopyrylium dye suitable for imaging in the red to near-infrared region. Also disclosed are pharmaceutical compositions and kits, as well as in vivo imaging methods, especially of use in the detection, staging, diagnosis, monitoring of disease progression or monitoring of treatment of colorectal cancer (CRC).
Field of the Invention

The present invention relates to labelled cMet binding peptides suitable for optical imaging. The peptides are labelled with an optical reporter group suitable for imaging in the red to near-infrared region. Also disclosed are in vivo imaging methods, especially of use in the diagnosis of colorectal cancer (CRC).

Background to the Invention

WO 2005/030266 discloses that there is a medical need for early diagnosis of colorectal cancer (CRC). WO 2005/030266 discloses optical imaging contrast agents which have affinity for a biological target abnormally expressed in CRC. The biological target is selected from COX-2, beta-catenin, E-cadherin, P-cadherin, various kinases, Hei-2, matrix metalloproteinases (MMPs), cyclophilins, P53, thymidylate synthase, VEGF receptor, EGF receptors, K-ras, adenomatous polyposis coli protein, cathepsin B, uPAR, cMet, mucins and gastric receptors. Preferred such targets are cMet, MMP-14, COX-2, beta-catenin and Cathepsin B.

The vectors of WO 2005/030266 can be a peptide, peptoid moiety, oligonucleotide, oligosaccharide, and related compound or traditional organic drug-like small molecule. The reporter moiety is preferably a dye that interacts with light in the wavelength region from the ultraviolet to the infrared part of the electromagnetic spectrum.

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a growth factor which is involved in various physiological processes, such as wound healing and angiogenesis. The HGF interaction with its high affinity receptor (cMet) is implicated in tumour growth, invasion and metastasis.

Knudsen et al. have reviewed the role of HGF and cMet in prostate cancer, with possible implications for imaging and therapy [Adv Cancer Res., 91, 31-67 (2004)]. Labelled anti-met antibodies for diagnosis and therapy are described in WO 03/057155.

WO 2004/078778 discloses polypeptides of multimeric peptide constructs which bind...
cMet of a complex comprising cMet and HGF. Approximately 10 different structural classes of peptide are described. WO 2004/078778 discloses that the peptides can be labelled with a detectable label for \textit{in utro} and \textit{in vivo} applications, or with a drug for therapeutic applications. The detectable label can be an enzyme, fluorescent compound, an optical dye, a paramagnetic metal ion, an ultrasound contrast agent or a gadtonuchde. Preferred labels of WO 2004/078778 are stated to be radioactive or paramagnetic, and most preferably comprise a metal which is chelated by a metal chelator.

The Present Invention

The present invention provides imaging agents suitable for \textit{in vivo} optical imaging, which comprise cMet binding cyclic peptides, and a benzopyryyhum dye suitable for imaging the mammalian body \textit{in vivo} using light of red to near-infrared wavelength 600-1200 nm.

The cMet binding cyclic peptides are related to one of the structural classes of peptide of WO 2004/078778, and have optimal binding affinity for cMet. These peptides were derived from phage display and selected by their affinity for cMet and lack of competition with HGF, as described in WO 2004/078778. The cMet binding peptides of the present invention preferably have at least one of their termini protected by metabolism inhibiting groups (M°). That is an important consideration for \textit{in vivo} applications, where endogenous enzymes and peptidases would otherwise rapidly metabolise the peptide, with consequent loss of cMet binding affinity, and hence loss of selective targeting \textit{in vivo}.

The present invention teaches that the best way of using cMet binding peptides for \textit{in vivo} imaging of superficial lesions involves the use of an optical reportei, as opposed to other imaging modalities (eg nuclear, MRI or ultrasound), and also provides preferred optical imaging reportei's. The ideal to near-infrared region (light of wavelength 600-1200 nm) is preferred, since that region has minimal spectral overlap with endogenous tissues and materials, such as haemoglobin, porphyryims, melanin, and collagen [Licha, Topics Cuπ.Chem, 222, 1-29 (2002)]. Other important contributors to tissue autofluorescence are NADH, FAD and elastin.
Detailed Description of the Invention

In a first aspect, the present invention provides an imaging agent which comprises a conjugate of Formula I

\[ Z^1\text{-}[\text{cMBP}].Z^2 \]

\[(L)_n[\text{Bz}]^M \]

(I)

where \( Z^1 \) is attached to the N-terminus of cMBP, and is \( H_01M^G \),

\( Z^7 \) is attached to the C-terminus of cMBP and is \( OH, OH_01M^D \),

where \( B^C \) is a biocompatible cation,

cMBP is a cMct binding cyclic peptide of 17 to 30 amino acids which

comprises the amino acid sequence (SEQ-I)

\[ \text{Cys}^a\cdot \text{X}^1\cdot \text{Cys}^a\cdot \text{X}^2\cdot \text{Gly}\cdot \text{Pro}\cdot \text{Pro}\cdot \text{X}^3\cdot \text{Phe}\cdot \text{Glu}\cdot \text{Cys}^d\cdot \text{Trp}\cdot \text{Cys}^b\cdot \text{Ty}^1\cdot \text{X}^4\cdot \text{X}^5\cdot \text{X}^6, \]

wherein \( X^1 \) is Asn, His or Tyr,

\( X^2 \) is Gly, Ser, Thr or Asn,

\( X^3 \) is Thr or Arg,

\( X^4 \) is Ala, Asp, Glu, Gly or Ser,

\( X^5 \) is Ser or Thr,

\( X^6 \) is Asp or Glu,

and Cys\(^d\) are each cysteine residues such that residues a and b as well as c and d are cyclised to form two separate disulfide bonds,

\( M^G \) is a metabolism inhibiting group which is a biocompatible group which inhibits or suppresses \textit{in vivo} metabolism of the peptide,

\( L \) is a synthetic hinge group of formula -(A)\(_m\) - wherein each A is independently -CR\(_x\), -CR=CR\(_x\), -C=C\(_x\), -CR\(_x\)CO\(_x\), -CO\(_x\)CR\(_x\), -NRCO\(_x\), -CONR\(_x\), -NR(C=O)NR\(_x\), -NR(C=SO\(_x\))NR\(_x\), -SO\(_x\)NR\(_x\), -NRSO\(_x\), -CR\(_x\)OCR\(_x\), -CR\(_x\)SCR\(_x\), -CR\(_x\)NCR\(_x\), a C\(_{4,5}\) cyclohexyloalkylene group, a C\(_{4,5}\) cycloalkylene group, a C\(_{4,5}\) alylenic group, or a C\(_{1,5}\) heteroalyleylene group, an amino acid, a sugar or a monodisperse polyethyleneglycol (PEG) building block,
each R is independently chosen from H, C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{1-4} alkoxyalkyl or C_{1-4} hydroxyalkyl;
m is an integer of value 1 to 20;
n is an integer of value 0 or 1;
Bzp^M is a benzopyrylium dye of Formula II:

(II)

where:

Y^1 is a group of Formula Y^d or Y^b

(Y^a)

(Y^b)

R'^{1-4} and R'^{9-13} are independently selected from H, -SO_{3}M^{1}, Hal, R^a or C_{3-12} aryl, where each M^{1} is independently H or B^{c}, and B^{c} is a biocompatible cation;
R^5 is H, C_{1-4} alkyl, C_{1-6} carboxyalkyl, C_{3-12} arylsulfonyl, Cl, or R^5 together with one of R^6, R^14, R^i^{5} or R^i^{6} may optionally form a 5- or 6- membered unsaturated aliphatic, unsaturated heteroaliphatic or aromatic ring;
R^6 and R^i^{6} are independently R^a groups;
R^7 and R^8 are independently C_{1-4} alkyl, C_{1-4} sulfoalkyl or C_{1-6} hydroxyalkyl or optionally together with one or both of R^9 and/or R^{10} may form a 5- or 6- membered N-containing heterocyclic or heteroaryl ring;
X is -CR^{14}R^{15-}, -O-, -S-, -Se-, -NR^{16}- or -CH=CH-, where R^{14} to R^{16} are independently R^a groups;
R^a is C_{1-4} alkyl, C_{3-4} sulfoalkyl, C_{1-6} carboxyalkyl or C_{1-6} hydroxyalkyl;
w is 1 or 2;
J is a biocompatible anion, with the proviso that B/pM comprises at least one sulfonic acid substituent chosen from the R¹ to R¹⁶ groups.

By the term "imaging agent" is meant a compound suitable for imaging the mammalian body \( w \); \( v \); \( n \); \( o \). Piefeiably, the mammal is a human subject. The imaging may be invasive (e.g., tomographic \( o \); \( l \) endoscopy) or non-invasive. The piefened imaging method is endoscopy. Whilst the conjugate of Formula I is suitable for \( \text{III} \) \( \text{vivo} \) imaging, it may also have \( \text{in} \); \( \text{vitro} \) applications (e.g., assays quantifying cMet in biological samples \( o \); \( l \) visualisation of cMet in tissue samples). Piefeiably, the imaging agent is used for \( \text{in} \); \( \text{vivo} \) optical imaging.

By the term "optical imaging" is meant any method that for\( \pi \)ns an image for detection, staging or diagnosis of disease, follow up of disease development \( o \); \( l \) for follow up of disease treatment based on interaction with light \( m \) the ied to neai-mfiaied region (wavelength 600-1200 nm). Optical imaging further includes all methods from dnect visualization without use of any device and involving use of devices such as various scopes, catheteis and optical imaging equipment, eg computei-assisted haidwaie for tomographic presentations. The modalities and measureiiment techniques include, but are not limited to luminescence imaging, endoscopy, fluoiescence endoscopy, optical coheience tomography, tiansmittance imaging, time lessolved tiansmittance imaging, confocal imaging, nonheai micoscopy, photoacoustic imaging, acousto-optical imaging, spectioscopy, ieflectance spectioscopy, mteifieiometry, coheience mteifieiometry, diffuse optical tomography and fluoiescence mediated diffuse optical tomography (continuous wave, time domain and frequency domain systems), and measurement of light scatteriiig, absiiption, polaiization, lumincescence, fluoiescence lifetime, quantum yield, and quenching. Further details of these techniques are provided by (Tuan Vo-Dmh (editor)) "Biomedical Photonics Handbook" (2003), CRC Press LCC, Mycek & Pogue (editoi) "Handbook of Biomedical Fluorescence" (2003), Maicel Dekkei, Inc., Sphteie & Hoppe "An Intoduction to Biomedical Optics" (2007), CRC Press LCC.

The ied to neai-mfiaied region light is piefeiably of wavelength 650-1000 nm. The optical imaging method is piefetably fluoiescence endoscopy.
By the term "conjugate" is meant that the cMBP and Bzp dye aie linked by covalent bonds, optionally via the \((L)\) moiety at the \(Z_1\) position gives compounds of formulae \([\text{Bzp M}]-(L)_n-\text{cMBP}-Z_2\) when the cMBP is pieffened.

The \(Z^1\) group substitutes the amine group of the last amino acid residue. Thus, when \(Z^1\) is H, the amine terminus of the cMBP teimmates in a free \(\text{NH}_2\) group of the last amino acid residue. The \(Z^2\) group substitutes the caibonyl group of the last amino acid residue. Thus, when \(Z^2\) is OH, the caibonyl teimmus of the cMBP terminates in the free \(\text{CO}_2\text{H}\) group of the last amino acid residue, and when \(Z^2\) is \(\text{OB}^2\) that terminal caibonyl group is ionised as a \(\text{CO}_2\text{B}^+\) caiboxylate group.

By the term "metabolism inhibiting group" (M\(^\text{II}\)) is meant a biocompatible group which inhibits \(\text{M}^\text{I}\) suppiess \(\text{III vivo}\) metabolism of the cMBP peptide at either the amine teimmus (\(Z^1\)) or caiboxyl terminus (\(Z^2\)). Such groups are well known to those skilled in the art and are suitably chosen from, for the peptide amine terminus:

N-acylated groups \(-\text{NH}(\text{C}=\text{O})\text{R}^0\) where the acyl group \((\text{C}=\text{O})\text{R}^0\) has \(\text{R}^0\) chosen from \(\text{Ci}_{6}\) alkyl, \(\text{C}_{10}\) ayl groups \(\text{M}^\text{I}\) composes a polyethyleneglycol (PEG) building block. Suitable PEG groups are described for the hneki group (L), below Piefened such PEG groups are the biomodifieis of Formula IA \(\text{M}^\text{I}\) IB. Piefened such ammoo terminus \(\text{M}^\text{II}\) groups are acetyl, benzyloxycaibonyl \(\text{M}^\text{I}\) fluioaoacetetyl, most piefeiably acetyl.

Suitable metabolism inhibiting groups for the peptide caiboxyl terminus include caiboxamid, \(\text{te}/\text{r}-\text{butyl estei}, \text{benzy estei}, \text{cyclohexyl estei}, \text{ammo alcohol \(\text{M}^\text{I}\)}\) a polyethyleneglycol (PEG) building block. A suitable \(\text{M}^\text{II}\) group for the caibonyl terminal amono acid residue of the cMBP peptide is wheie the terminal amine of the amono acid residue is N-alkylated with a \(\text{Ci}_{4}\) alkyl group, piefetably a methyl group. Piefened such \(\text{M}^\text{II}\) groups are caiboxamide \(\text{M}^\text{I}\) PEG, most piefened such groups are caiboxamide.

Formula I denotes that the \((L)_n[\text{Bzp M}]\) moiety can be attached at either \(Z^1, Z^2\) \(\text{cMBP}\) for \(Z^1\) \(\text{M}^\text{I}\) \(Z^2\), the \((L)_n[\text{Bzp M}]\) moiety may either be attached to the \(\text{M}^\text{II}\) group when either of \(Z/Z^2\) is \(\text{M}^\text{II}\). When \(Z^1\) is \(\text{H}\) \(\text{M}\) \(Z^2\) is \(\text{OH}\), attachment of the \((L)_n[\text{Bzp M}]\) moiety at the \(Z^1\) \(\text{M}\) \(Z^2\) position gives compounds of formulae \([\text{Bzp M}]-\text{(L)_n-cMBP}]-Z^2\).
ot $Z^-[\text{cMBP}](L)_n[Bzp^M]$ respectively. Inhibition of metabolism of the cMBP at either peptide terminus may also be achieved by attachment of the $(L)_n[Bzp^\eta]$ moiety in this way, but $(L)_n[Bzp^\eta]$ is outside the definition of $M^K$ of the present invention.

The $(L)_n^-$ moiety of Formula I may be attached at any suitable position of the Bzp$^M$ of Formula II. The $(L)_n^-$ moiety either takes the place of an existing substituent (e.g. one of the $R^1$ to $R^{16}$ groups), or is covalently attached to the existing substituent of the Bzp$^M$. The $(L)_n^-$ moiety of Formula I is preferably attached via a carboxyalkyl substituent of the Bzp$^M$.

By the term "cMet binding cyclic peptide" (cMBP) is meant a peptide which binds to the hepatocyte growth factor (HGF) high affinity receptor, also known as cMet (c-Met, Met, Met receptor or hepatocyte growth factor receptor). Suitable cMBP peptides of the present invention have an apparent $K_d$ for cMet of cMet/HGF complex of less than about 20 nM. The cMBP peptides comprise pioline residues, and it is known that such residues can exhibit cis/trans isomerisation of the backbone amide bond. The cMBP peptides of the present invention include any such isomers.

By the term "biocompatible cation" (B$^c$) is meant a positively charged counterion which forms a salt with an ionised, negatively charged group, where said positively charged counterion is also non-toxic and hence suitable for administration to the mammalian body, especially the human body. Examples of suitable biocompatible cations include the alkali metals sodium or potassium, the alkaline earth metals calcium and magnesium, and the ammonium ion. A preferred biocompatible cation is sodium and potassium, most preferably sodium. By the term "biocompatible anion" (J) is meant a negatively charged counterion which forms a salt with an ionised, negatively charged group (in this case an ammonium group), where said negatively charged counterion is also non-toxic and hence suitable for administration to the mammalian body, especially the human body. The counterion (J) represents an anion which is present in a molal equivalent amount, thus balancing the positive charge on the Bzp$^\eta$ dye. The anion (1) is suitably singly- or multiply-charged, as long as a charged-balancing amount is present. The anion is suitably derived from an amino acid or an amino acid analogue. Examples of suitable anions include halide ions such as chloride or biomide, sulfate, nitrate, citrate, acetate, phosphate and boiote. A preferred anion is...
chloride

By the term "amino acid" is meant an L- or D- amino acid, amino acid analogue (e.g., naphthylalanine) or amino acid mimetic which may be naturally occurring or of purely synthetic origin, and may be optically pure, i.e., a single enantiomer and hence chiral, or a mixture of enantiomers. Conventional 3-letter or single letter abbreviations for amino acids are used here. Preferably the amino acids of the present invention are optically pure. By the term "amino acid mimetic" is meant synthetic analogues of naturally occurring amino acids which are isosteres, i.e., have been designed to mimic the steric and electronic structure of the natural compound. Such isosteres are well known to those skilled in the art and include but are not limited to depsipeptides, retro-mverso peptides, thioamides, cycloalkanes or 1,5-disubstituted tetrazoles [see M. Goodman, Biopolymeis, 24, 137, (1985)].

By the term "peptide" is meant a compound comprising two or more amino acids, as defined above, linked by a peptide bond (i.e., an amide bond linking the amine of one amino acid to the carboxyl of another). The term "peptide mimetic" or "mimetic" refers to biologically active compounds that mimic the biological activity of a peptide of a protein but are no longer peptidic in chemical nature, that is, they no longer contain any peptide bonds (that is, amide bonds between amino acids). Here, the term peptide mimetic is used in a broader sense to include molecules that are no longer completely peptidic in nature, such as pseudo-peptides, semi-peptides and peptoids.

Suitable imaging agents of the invention are those wherein the Bzp is of Formula Ha or lib.
When $R^3$ together with one of $R^6/R^{14}-R^{16}$ forms a 5-oi 6-membered unsaturated aliphatic, unsaturated heteroaliphatic or aromatic ring, suitable such aromatic rings include phenyl, fuian, thiazole, pyndyl, pyrene or pyrazole rings. Suitable unsaturated rings comprise at least the C=C to which $R^3$ is attached.

When $R^7$ and/or $R^8$ together with one of both of $R^9$ and/or $R^{10}$ form a 5-oi 6-membered N-containing heterocyclic or heteroaryl ring, suitable such rings include thiazole, pyndyl, pyrene or pyrazole rings or partially hydrogenated versions thereof.

By the term "sulfonic acid substituent" is meant a substituent of formula $-\text{SO}_3\text{H}$, where $\text{H}$ is $\text{H}^\text{c}$, and $\text{B}^\text{c}$ is a biocompatible cation (as defined above). The $-\text{SO}_3\text{H}$ substituent is covalently bonded to a carbon atom, and the carbon atom may be aiyl (i.e., sulfoaiyl such as when $R^1$ or $R^2$ is $-\text{SO}_3\text{M}^\text{c}$), or alkyl (i.e., sulfoalkyl group).

It is envisaged that one of the roles of the lankei group $-(\text{A})_n$ of Formula I is to distance Bzp$^\text{M}$ from the binding site of the cMBP peptide, to minimise any steric impairment of interaction with the binding site. This can be achieved by a combination of flexibility (e.g., simple alkyl chains), so that the bulky group has the freedom to position itself away from the active site and/or rigidity such as a cycloalkyl or aiyl spacer which orientate the Bzp$^\text{M}$ away from the binding site. The nature of the lankei group can also be used to modify the biodistribution of the imaging agent. Thus, e.g., the introduction of ethet groups in the lankei will help to minimise plasma protein...
binding. When -(A) \(_n\) comprises a polyethyleneglycol (PEG) building block and a peptide chain of 1 to 10 amino acid residues, the hinge group may function to modify the pharmacokinetics and blood clearance rates of the imaging agent \textit{in vivo}. Such "biomodified" hinge groups may accelerate the clearance of the imaging agent from background tissue, such as muscle or liver, and/or from the blood, thus giving a better diagnostic image due to less background interference. A biomodified hinge group may also be used to favor a particular route of excretion, e.g., \textit{via} the kidneys as opposed to \textit{via} the liver.

By the term "sugar" is meant a mono-, di-, or \(\tau\)-saccharide. Suitable sugars include glucose, galactose, maltose, mannose, and lactose. Optionally, the sugar may be functionalized to permit facile coupling to amino acids. Thus, e.g., a glucosamine derivative of an amino acid can be conjugated to other amino acids \textit{via} peptide bonds. The glucosamine derivative of aspartagine (commercially available from NovaBiochem) is one example of this.

Piefered features

The molecular weight of the imaging agent is suitably up to 8000 Daltons. Pieferably, the molecular weight is in the range 2800 to 6000 Daltons, most pieferably 3000 to 4500 Daltons, with 3200 to 4000 Daltons being especially pieferened.

Piefered imaging agents of the present invention have both peptide termini protected by M\(^{10}\) groups, i.e., pieferably both \(Z^1\) and \(Z^2\) are M\(^{10}\), which will usually be different. As noted above, either of \(Z^1/Z^2\) may optionally represent -(L)\(_n\)[Bzp\(^{M}\)]. Having both peptide termini protected in this way is important for \textit{in vivo} imaging applications, since otherwise rapid metabolism would be expected with consequent loss of selective...
binding affinity for cMet. When both Z₁ and Z₂ are $M^\text{ac}$, preferably Z₁ is acetyl and Z₂ is a primary amide. Most preferably, Z₁ is acetyl and Z₂ is a primary amide and the (L)$_n$[Bzp]$^M$ moiety is attached to the epsilon amine side chain of a lysine residue of cMBP.

Piefeired cMBP peptides of the present invention have a $K_i$ for binding to cMet of less than about 10 nM (based on fluorescence polarization assay measurements), most preferably less than 5 nM, with less than 3 nM being the ideal.

The peptide sequence (SEQ-I)

$$\text{Cys}^d\text{X}^1\text{-Cys}^c\text{-X}^2\text{-Gly-Pio-Pio-X}'\text{-Phe-Glu-Cys}^d\text{-Trp-Cys}^b\text{-Tyi-X}^4\text{-X}''\text{-X}^6$$

of the cMBP of Formula I is a 17-mei peptide sequence, which is piimanly responsible for the selective binding to cMet. When the cMBP peptide of the present invention comprises more than 17 amino acid residues, the peptidometh amino acids can be any amino acid apart from cysteine. Additional, unprotected cysteine residues could cause unwanted scrambling of the defined Cys$^a$-Cys$^b$ and Cys$^c$-Cys$^d$ disulfide bridges. The additional peptides preferably comprise at least one amino acid residue with a side chain suitable for facile conjugation of the (L)$_n$[Bzp]$^M$ moiety. Suitable such residues include Asp or Glu residues for conjugation with amine-functionalised -(L)$_n$Bzp$^M$ groups, or a Lys residue for conjugation with an amine-functionalised -(L)$_n$Bzp$^M$ group. The amino acid residues for conjugation of -(L)$_n$[Bzp]$^M$ are suitably located away from the 17-mei binding region of the cMBP peptide (SEQ-I), and are preferably located at the C- or N-terminus. Preferably, the amino acid residue for conjugation is a Lys residue.

Substitution of the tryptophan residue of SEQ-I was evaluated with the known amino acid substitutes phenylalanine and naphthylalanine. Loss of cMet affinity was, however, found suggesting that the tryptophan residue is important for activity.

It is piefeired that the cMBP peptide further comprises a N-terminal senne residue, giving the 18-mei (SEQ-2)

$$\text{Sel-Cys}^a\text{-X}^1\text{-Cys}^l\text{-X}^2\text{-Gly-Pio-Pio-X}'\text{-Phe-Glu-Cys}^d\text{-Tip-Cys}^b\text{-Tyi-X}^4\text{-X}''\text{-X}^i$$

In addition to SEQ-I, or preferably SEQ-2, the cMBP most preferably further
comp\(\pi\)ses eith ei

(i) an Asp or Glu residue within 4 amino acid residues of eith ei the C- 0 1 N- peptide terminus, and \((L)_nBzp^{3l}\) is functionahsed with an amine gioup which is conjugated to the caiboxyl side chain of said Asp or Glu isidue to give an amide bond,

(ii) a Lys isidue withm 4 amino acid isidues of eith ei the C- oi N- peptide terminus, and \((L)_nBzp^M\) is functionahsed with a caiboxyl gioup which is conjugated to the epsilon amine side chain of said Lys isidue to give an amide bond

Picfe\(\pi\)ed cMBP peptides comprise the 22-mei ammoe acid sequence (SEQ-3)

\[
\text{Ala-Gly-Sei-Cys}^{a}\cdot X'\cdot \text{Cys}^{c}\cdot X^2\cdot \text{Gly-Pio-Pio-X}^{3}\cdot \text{Phe-Glu-Cys}^{d}\cdot T\phi \cdot \text{Cys}^{b}\cdot \text{Tyi-} \\
X^4\cdot X'\cdot X^6\cdot \text{Gly-Tht}
\]

The cMBP peptides of the piesen invention pifieably have \(X^3\) equal to \(Aig\)

The cMBP peptide pifieably fuithei comprises in addition to SEQ-I, SEQ-2 ot SEQ-3, at eith ei the N- oi C- terminus a hnkei peptide which is chosen fiom

\[-\text{Gly-Gly-Gly-Lys-} \quad (\text{SEQ-4}), \quad -\text{Gly-Sei-Gly-Lys-} \quad (\text{SEQ-5}) \text{ oi}
\]

\[-\text{Gly-Sei-Gly-Sei-Lys-} \quad (\text{SEQ-6})
\]

The Lys isidue of the hnkei peptide is a most piefened location foi conjugation of the \(-(L)_n[Bzp^M]\) moiety Especially piefened cMBP peptides comprise SEQ-3 togethei with the hnkei peptide of SEQ-4, having the 26-mei ammoe acid sequence (SEQ-7)

\[
\text{Ala-Gly-Sei-Cys}^{a}\cdot \text{Tyi-Cys}^{c}\cdot \text{Sei-Gly-Pio-Pio-Aig-Phe-Glu-Cys}^{d}\cdot \text{T\phi-Cys}^{b}\cdot \\
\text{Tyi-Glu-Tht-Glu-Gly-Thi-Gly-Gly-Gly-Lys}
\]

cMBP peptides of SEQ-I, SEQ-2, SEQ-3 and SEQ-7 pifieably have \(Z^1 = Z^2 = M^K\), and most pifieably have \(Z^1 = \text{acetyl and } Z^2 = \text{pnmaiy amide}

The \(-(L)_n[Bzp^{vl}]\) moiety is suitably attached to eith ei of the \(Z^1\) oi \(Z^2\) gioups oi an ammoe acid isidue of the cMBP peptide which is diffeient to the cMet binding sequence of SEQ-I Pife\(\pi\)ed ammoe acid isidues and sites of conjugation aie as descriibed above When the \(-(L)_n[Bzp^M]\) moiety is attached to \(Z^1\) oi \(Z^2\), it may take
the place of $Z^1_0 Z^2$ by conjugation to the N- or C- terminus, and block *in vivo* metabolism in that way.

The [Bzp$^M$]-(L)$_n$- moiety of Formula I is preferably attached at positions $R^6$, $R^{14}$, $R^{15}$ or $R^{16}$ of the Bzp$^M$ of Formula II, moie preferably at $R^6$, $R^{14}$, $R^{15}$ or $R^{16}$ most preferably at $R^6$, $R^{14}$ or $R^{15}$. In oedei to facilitate the attachment, the relevant $R^6$, $R^6$, $R^{14}$, $R^{15}$ or $R^{16}$ substituent is preferably $C_{16}$ caiboxyalkyl, more preferably $C_{14}$ caiboxyalkyl.

The beiVopyiylmm dye (Bzp$^M$) preferably has at least 2 sulfonic acid substituents, moic preferably 2 to 6 sulfonic acid substituents, most preferably 2 to 4 sulfonic acid substituents. Preferably, at least one of the sulfonic acid substituents is a $C_{14}$ sulfoalkyl group. Such sulfoalkyl groups aie preferably located at positions $R^6$, $R^7$, $R^8$, $R^{14}$, $R^{15}$ or $R^{16}$, moie preferably at $R^6$, $R^7$, $R^8$, $R^{16}$ or $R^{16}$ most preferably at $R^6$ togethci with one or both of $R^7$ and $R^8$ of Formula II. The sulfoalkyl groups of Formula II, aie preferably of formula $-(CH_2)_xSO M^1$, wheie $M^1$ is $H$, $B^c$, $k$ is an integei of value 1 to 4, and $B^c$ is a biocompatible cation (as defined above). $k$ is preferably 3 to 4.

In Formula II, $w$ is preferably 1. $R^7$ is preferably $H$, $C_{14}$ caiboxyalkyl, and is most preferably $C_{14}$ caiboxyalkyl, and is most preferably $H$, $X$ is preferably $-CR^{14}R^{15}$, and is most preferably $-CR^{14}R^{15}$.

Prefered Bzp$^M$ dyes aie of Formula III.

![Diagram](III)

 wheie $Y^1$, $R^1$, $R^2$, $R^3$, $R^4$, $R^6$, $R^{14}$, $R^{15}$ and $I$ ate as defined foi Formula II.

Suitable dyes of Formula III aie of Formula II.
Preferred $R^1$-$R^4$ and $R^6$-$R^{13}$ groups of Formulae III, Ilia and IHb are as described above for Formulae Ha and lib. In Formulae III, Ilia and IHb, $R^{14}$ and $R^{15}$ are preferably chosen such that one is an $R^b$ group and the other is an $R^c$ group. $R^b$ is $C_{1-2}$ alkyl, most preferably methyl. $R^c$ is $C_{1-4}$ alkyl, $C_{3-6}$ carboxyalkyl or $C_{1-4}$ sulfoalkyl, preferably $C_{3-6}$ carboxyalkyl or $-(CH_2)_kXSChM^1$ where $k$ is chosen to be 3 or 4.

Preferably the dyes of Formula III have a $C_{1-6}$ carboxyalkyl substituent to permit facile covalent attachment to the cMBP.

In Formula II or III, when $R^7$ and/or $R^8$ together with one or both of $R^9$ and/or $R^{10}$ form a 5- or 6-membered N-containing heterocyclic or heteroaryl ring, preferred such rings are pyridyl or dihydropyridyl. A preferred such $Y^1$ group wherein an $R^8$ group has been cyclised with $R^{10}$ is of Formula $Y^c$:  

$$
\begin{array}{c}
\text{(Ilia)}
\end{array}
$$

$$
\begin{array}{c}
\text{(IHb)}
\end{array}
$$
A piefe π ed such Y¹ gioup wheiein both R⁷ and R⁸ gioup have been cyclised is of Formula Y¹

(Y¹)

R⁷, R⁹ and R¹¹-R¹³ ate as defined above,

each E¹ is independently H or Cᵣ₄ alkyl

In Formula Y¹ it is piefe π ed that
each X¹ is OHU,
R⁹ = Rπ = H,
Rₚ is H,
R¹² is CH₃ 0₁ -C(CH₃)₃K moie piefeiably -C(CHOi)

Yd

(Yd)

In Forimula Yd, it is piefe π ed that
R⁹ = H,
R¹² is H,
R⁰ is piefeiably CH, ot -C(CHOs, moie piefeiably -C(CHOi)

It is piefe π ed that the -NR⁷R⁸ gioup of Formula III is either

(1) in open chain foim, ie the R⁷/R⁸ gioups are not cyclised with one or both of R⁹/R¹₀. Piefe π ed such R⁷ and R⁸ gioups are independently chosen fiom Cᵣ₄ alkyl 0₁ Cᵣ₄ sulfoalkyl, most piefeiably ethyl 0₁ Cᵣ₄
sitlfoalkyl.

(u) cyclised to give a cyclic \( Y^1 \) substituent of Formula \( Y^c \) or \( Y^d \), moie preferably of Formula \( Y^c \).

The open chain form (i) is most pieferred

Especially preferred dyes of Formula III are of Formula Hlc, IIId or IHe

\[
\text{(Hlc)}
\]

\[
\text{(IIIId)}
\]

\[
\text{(IHe)}
\]

where:

- \( M^1 \) is as defined above,
- \( R^{17} \) and \( R^{18} \) are independently chosen from \( C_1 \) 4 alkyl or \( C_1 \) 4 sulfoalkyl,
- \( R^{19} \) is \( H \) or \( C_4 \) alkyl,
R\textsuperscript{20} is C\textsubscript{i-4} alkyl, C\textsubscript{i-4} sulfoalkyl or C\textsubscript{i-6} carboxyalkyl; 
R\textsuperscript{21} is C\textsubscript{i-4} sulfoalkyl or C\textsubscript{i-6} carboxyalkyl; 
R\textsuperscript{22} is C\textsubscript{i-4} alkyl, C\textsubscript{i-4} sulfoalkyl or C\textsubscript{i-6} carboxyalkyl; 
E\textsuperscript{2}, E\textsuperscript{3} and E\textsuperscript{4} are independently H or C\textsubscript{i-1} alkyl.

The dyes of Formulae IHd, IHe and IHf are preferably chosen such that one or more of \( R^{19} \)-\( R^{22} \) is C\textsubscript{i-4} sulfoalkyl.

Preferred specific dyes of Formula Hid are DY-63 1 and DY-633:

\begin{center}
\includegraphics{dy63_1.png}
\end{center}

DY-63 1

\begin{center}
\includegraphics{dy63_3.png}
\end{center}

DY-633

A preferred specific dye of Formula Hie is DY-652:
Preferred specific dyes are DY-631 and DY-652, with DY-652 being most preferred.

When a synthetic linker group (L) is present, it preferably comprises terminal functional groups which facilitate conjugation to $[\text{Bzp}^\lambda \text{J}]$ and $Z'-[\text{cMBP}^\text{I}-\text{Z}^\text{Z}]$. When L comprises a peptide chain of 1 to 10 amino acid residues, the amino acid residues are preferably chosen from glycine, lysine, arginine, aspartic acid, glutamic acid or serine. When L comprises a PEG moiety, it preferably comprises units derived from oligomerisation of the monodisperse PEG-like structures of Formulae Biol or Bio2:

$$\text{(Biol)}$$

17-amino-5-oxo-6-aza-3, 9, 12, 15-tetraoxaheptadecanoic acid of Formula Biol

wherein $p$ is an integer from 1 to 10. Alternatively, a PEG-like structure based on a propionic acid derivative of Formula Bio2 can be used:

$$\text{(Bio2)}$$

where $p$ is as defined for Formula Biol and $q$ is an integer from 3 to 15.
In Formula Bio2, p is preferably 1 or 2, and q is preferably 5 to 12.

When the linker group does not comprise PEG or a peptide chain, preferred linker groups have a backbone chain of linked atoms which make up the \( -(A)_m \) moiety of 2 to 10 atoms, most preferably 2 to 5 atoms, with 2 or 3 atoms being especially preferred. A minimum linker group backbone chain of 2 atoms confers the advantage that the imaging moiety is well-separated so that any undesired interaction is minimised.

In Formula I, n is preferably 0 or 1, most preferably 0, i.e., no linker group is present.

Preferred imaging agents of the present invention are of Formula IV:

\[
\begin{align*}
&\text{M}^{\text{IG}}: \text{Ala-Gly-Sei-Cys}^a : \text{Tyr-Cys}^c : \text{Sei-Gly-Pi o-Pro-Arg-Phe-Glu-Cys}^d : \text{Trp-}

&\text{Cys}^b : \text{Tyr-Glu-Thi-Glu-Gly-Thr-Gly-Gly-Gly-Lys-M}^r

&\( (L)_n [Bzp^M] \)
\end{align*}
\]

wherein the \( (L)_n [Bzp^M] \) group is attached to the epsilon amino group of the Lys residue. Preferred imaging agents of Formula IV have \( \text{M}^{\text{IG}} \) (N-terminal Ala) equal to acetyl and \( \text{M}^{\text{IG}} \) (C-terminal Lys) equal to primary amide. In Formula IV, n is preferably zero and Bzp\(^M\) is preferably of Formula IUA or IHb, more preferably of Formula IHc, IHd or IHe, most preferably the specific dyes DY-63 1, DY-633 or DY-652. In Formula IV, the most preferred specific dye is DY-652.

Peptides of formula \( Z':[cMBP]-Z \)^2 of the present invention may be obtained by a method of preparation which comprises:

(i) solid phase peptide synthesis of a linear peptide which has the same peptide sequence as the desired cMBP peptide and in which the Cys\(^a\) and Cys\(^b\) are unprotected, and the Cys\(^c\) and Cys\(^d\) residues have thiol-protection groups,

(ii) treatment of the peptide from step (i) with aqueous base in solution to give a monocyclic peptide with a first disulfide bond linking Cys\(^a\) and Cys\(^b\),

(iii) removal of the Cys\(^c\) and Cys\(^d\) thiol-protection groups and cyclisation to give a second disulfide bond linking Cys\(^c\) and Cys\(^d\), which is the desired bicyclic peptide product \( Z':[cMBP]-Z \)^2.
By the term "protecting group" is meant a group which inhibits undesired chemical reactions, but which is designed to be sufficiently inactive that it may be cleaved from the functional group in question under mild enough conditions that do not modify the rest of the molecule. After deprotection the desired product is obtained. Amine protecting groups are well known to those skilled in the art and are suitably chosen from Boc (where Boc is tert-butyloxycarbonyl), Fmoc (where Fmoc is fluorenlymethoxy carbonyl), tiifluoroacetyl, allyloxycarbonyl, Dde [i.e., 1-(4,4-dimethyl-2,6-dioxocyclohexyne)ethyl] or Npys (i.e., 3-mtio-2-pyridine sulfenyl). Suitable thiol protecting groups are Tt (Tπyl), Acm (acetamidomethyl), /-Bu (tert-butyl), /e/Z-Butylthio, methoxybenzyl, methylbenzyl or Npys (3-nitio-2-pyridine sulfenyl). The use of further protecting groups are described in "Protective Groups in Organic Synthesis", Theoioida W. Gieene and Petei G. M. Wuts, (John Wiley & Sons, 1991). Piefened amine protecting groups are Boc and Fmoc, most piefeiably Boc. Piefened amine protecting groups are Tt and Acm.

Examples 1 and 2 provide further specific details. Further details of solid phase peptide synthesis are described in P. Lloyd-Williams, F. Albeicico and E. Gnaid, Chemical Approaches to the Synthesis of Peptides and Proteins, CRC Press, 1997. The cMBP peptides are best stored undei inert atmosphere and kept in a freeze. When used in solution, it is best to avoid pH above 7 since the sks sciamblmg of the disulfide budes.

The imaging agents can be piepaied as described in the thud aspect (below).

In a second aspect, the present invention provides a pharmaceutical composition which composes the imaging agent of the first aspect together with a biocompatible cai πei, in a form suitable for mammalian administration.

The "biocompatible cai πei" is a fluid, especially a liquid, in which the imaging agent can be suspended or dissolved, such that the composition is physiologically tolerable, i.e., can be administered to the mammalian body without toxicity or undue discomfort. The biocompatible cai πei is suitably an injectable cai πei liquid such as sterile, pyrogen-free water for injection, an aqueous solution such as saline (which may
advantageously be balanced so that the final product for injection is isotonic), an aqueous solution of one 0.1 molar tonicity-adjusting substances (e.g., salts of plasma cations with biocompatible excipients), sugars (e.g., glucose 0.1 sucrose), sugars alcohols (e.g., sorbitol 0.1 mannitol), glycols (e.g., glycerol), 0.1 other non-ionic polyol mateials (e.g., polyethylene glycols, piopylene glycols and the like) Preferably the biocompatible carrier is pyrogen-free water for injection or isotonic saline.

The imaging agents and biocompatible camel are each supplied in suitable vials or vessels which comprise a sealed container which permits maintenance of sterile integrity, plus optionally an inextensible headspace gas (e.g., nitrogen or argon), whilst permitting addition and withdrawal of solutions by syringe or cannula. A prefilled such container is a septum-sealed vial, wherein the gas-tight closure is tampered on with an over-seal (typically of aluminium). The closure is suitable for single or multiple punctuations with a hypodermic needle (e.g., a ctdmped-on septum seal closure) whilst maintaining sterile integrity. Such containers have the additional advantage that the closure can withstand vacuum if desired (e.g., to change the headspace gas or degas solutions), and withstand pressure changes such as reductions in pressure without permitting ingress of external atmospheric gases, such as oxygen or water vapour.

Prefilled multiple dose containers comprise a single bulk vial (e.g., of 10 to 30 cm³ volume) which contains multiple patient doses, whereby single patient doses can thus be withdrawn into clinical grade syringes at various time intervals during the viable lifetime of the preparation to suit the clinical situation. Prefilled syringes are designed to contain a single human dose, or "unit dose" and are therefore preferably disposable or other syringe suitable for clinical use. The pharmaceutical compositions of the present invention preferably have a dosage suitable for a single patient and are provided in a suitable syringe or container, as described above.

The pharmaceutical composition may optionally contain additional excipients such as an antimicrobial preservative, pH-adjusting agent, filler, stabiliser or osmolality adjusting agent. By the term "antimicrobial preservative" is meant an agent which inhibits the growth of potentially harmful micro-organisms such as bacteria, yeasts or moulds. The antimicrobial preservative may also exhibit some bactericidal properties, depending on the dosage employed. The main role of the antimicrobial preservative(s)
of the present invention is to inhibit the growth of any such micro-organisms in the pharmaceutical composition. The antimicrobial pseivative may, however, also optionally be used to inhibit the growth of potentially harmful micro-organisms in one or more of the kits used to prepare said composition prior to administration.

Suitable antimicrobial pseervative(s) include the paeabens, i.e., methyl, ethyl, propyl, butyl paeaben or mixtures thereof, benzyl alcohol, phenol, ciesol, cetimide and thulomeisal. Preferred antimicrobial pseervative(s) are the paeabens.

The term "pH-adjusting agent" means a compound or mixture of compounds useful to ensure that the pH of the composition is within acceptable limits (approximately pH 4.0 to 10.5) for human or mammalian administration. Suitable such pH-adjusting agents include pharmaceutically acceptable buffers, such as tiicme, phosphate or TRIS [i.e., /m(hydroxymethyl)ammonomethane], and pharmaceutically acceptable bases such as sodium carbonate, sodium bicarbonate or mixtures thereof. When the composition is employed in kit form, the pH adjusting agent may optionally be provided in a separate vial or container, so that the use of the kit can adjust the pH as part of a multi-step procedure.

By the term "filler" is meant a pharmaceutically acceptable bulking agent which may facilitate material handling during production and lyophilisation. Suitable fillers include inorganic salts such as sodium chloide, and water soluble sugars such as sucrose, maltose, mannmol or tiehalose.

The pharmaceutical compositions of the second aspect may be prepared under aseptic manufacturing conditions to give the desired sterile, non-pyogenic product. It is preferred that the key components, especially the associated agents and those pairs of the apparatus which come into contact with the imaging agent (e.g., vials) are sterile. The components and agents can be sterilised by methods known in the art, including sterile filtration, terminal steihsation using e.g., gamma-irradiation, autoclaving, dry heat or chemical treatment (e.g., with ethylene oxide). It is preferred to sterilise some components in advance, so that the minimum number of manipulations needs to be carried out. As a precaution, however, it is preferred to include at least a sterile filtration step as the final step in the preparation of the
pharmaceutical composition

The pharmaceutical composition of the second aspect may optionally be piepaied from a kit, as described for the fourth aspect below.

In a thud aspect, the present invention provides a method of piepaiation of the imaging agent of the first aspect, which comprises one of steps (i) to (iv)

(i) reaction of a peptide of formula \( Z^{1} \text{cMBP} \cdot Z^{2} \) wheiein \( Z^{1} \) is \( H \) and \( Z^{2} \) is a \( M^{\text{n}} \) with a compound of formula \( j^{\prime}-(L)_{n} \cdot [\text{Bzp}^{M}] \), to give the imaging agent of Formula (I) wheiein \( \text{Bzp}^{M} \) is conjugated at the \( Z^{1} \) position,

(n) reaction of a peptide of formula \( Z^{1} \cdot [\text{cMBP}] \cdot Z^{2} \) wheiein \( Z^{1} = Z^{2} = M^{\text{v}} \) and cMBP comprises an Asp or Glu residue withm 4 amino acid residues of eithei the C- or N- cMBP peptide terminus, and all other Asp/Glu residues of the cMBP peptides are protected, with a compound of formula

\( \text{I}^{\prime}-(L)_{n} \cdot [\text{Bzp}^{M}] \), to give the imaging agent of Formula (I) wheiein \( \text{Bzp}^{M} \) is conjugated at said Asp or Glu residue of the cMBP peptide,

(in) reaction of a peptide of formula \( Z^{1} \cdot [\text{cMBP}] \cdot Z^{2} \) wheiein \( Z^{1} = Z^{2} = M^{\text{v}} \) and \( Z^{1} \) is a \( \gamma^{2} \) group of an activated ester and all other Asp/Glu residues of the cMBP peptides are protected, with a compound of formula

\( \text{I}^{\prime}-(L)_{n} \cdot [\text{Bzp}^{M}] \), to give the imaging agent of Formula (I) wheiein \( \text{Bzp}^{M} \) is conjugated at the \( Z^{1} \) position,

(iv) reaction of a peptide of formula \( Z^{1} \cdot [\text{cMBP}] \cdot Z^{2} \) wheiein \( Z^{1} = Z^{2} = M^{\text{v}} \) and cMBP comprises a Lys withm 4 amino acid residues of eithei the C- or N- cMBP peptide terminus, with a compound of formula \( \text{I}^{\prime}-(L)_{n} \cdot [\text{Bzp}^{M}] \), to give the imaging agent of Formula (I) wheiein \( \text{Bzp}^{M} \) is conjugated at a Lys residue of the cMBP peptide,

where

\( Z^{1} \), cMBP, \( Z^{2} \), \( M^{\gamma} \), \( L \), \( n \) and \( \text{Bzp}^{M} \) aie as defined in the first aspect,

\( Z^{1} \) is a \( \gamma^{2} \) group of an activated ester,

\( \text{T}^{1} \) is a carboxylic acid, activated ester, isothiocyanate or thiocyanate group,

\( \text{I}^{2} \) is an amine group

By the term "activated ester" or "active ester" is meant an ester derivative of the
carboxylic acid which is designed to be a better leaving group, and hence permit more facile interaction with nucleophiles, such as amines. Examples of suitable active esters are N-hydroxysuccinimide (NHS), pentafluorophenol, pentafluorothiophenol, para-nitrophenol and hydroxybenzotriazole. Preferred active esters are N-hydroxysuccinimide or pentafluorophenol esters.

J is preferably a primary or secondary amine group, most preferably a primary amine group.

The compound Z'-[cMBP]-Z^2 preferably has both Z^1 and Z^2 equal to M^G. Preferred cMBP peptides and Z'/Z^2 groups are as described in the first aspect. In particular, it is preferred that the cMBP peptide comprises an Asp, Glu or Lys residue to facilitate conjugation as described for the preferred cMBP peptides of the first aspect. It is especially preferred that the cMBP peptide comprises a Lys residue, as described in step (iv).

The preparation of the Z'-[cMBP]-Z^2 is described in the first embodiment (above). The Z'-[cMBP]-Z^2 peptide where Z^1 is an active ester can be prepared from Z'-[cMBP]-Z^2, where Z^2 is OH or a biocompatible cation (B^+), by conventional methods.

For conjugation with the cMBP, the Bzp^M suitably comprises a reactive or functional group (G) group which reacts with a complementary group of the cMBP peptide forming a covalent linkage between the dye and the cMBP peptide. G may be a reactive group (Q^a) that may react with a complementary functional group of the peptide, or alternatively may include a functional group that may react with a reactive group of the cMBP peptide. Examples of reactive and functional groups include active esters, isothiocyanate, malcimide, haloacetamide, acid halide, hydrazide, vinylsulfone, dichlorotriazole, phosphoamidite, hydroxyl, amno, sulfdryl, carbonyl, carboxylic acid and thiophosphate. Preferably G is a reactive group (Q^a). Q^a is preferably an active ester.

Benzopyrylium dyes (Bzp^M) functionalised suitable for conjugation to cMBP are
commericially available from Dyomics (Dyomics GmbH, Wmzeilaei St 2A, D-07745 Tena, Germany, www.dyomics.com), where the leactive group (Q²) is NHS ester, maleimide, amino 01 caioboxyh acid Picruisoi, suitable for the synthesis of benzopyiyhum dyes can also be piepaied as descibed in US 5405976 Methods of conjugating optical tepoiici dyes, to amino acids and peptides are descibed by Licha [Topics Cuπ Chem, 222, 1-29 (2002), Adv Diug Dev Rev, 57, 1087-1108 (2005)], as well as Flanagan et al [Bioconj Chem, 8, 751-756 (1997)], Li et al, ibid, 1.3, 605-610 (2002) and Zaheei [Mol Imaging, 1(4), 354-364 (2002)] Methods of conjugating the hkei gioup (L) to the cMBP peptide use analogous chemisty to that of the dyes alone (see above), and are known in the art

In a fourth aspect, the present invention provides a kit for the piepaiation of the pharmaceutical composition of the second aspect, which compises the imaging agent of the first aspect in stenle, solid form such that, upon ieconstitution with a stenle supply of the biocompatible caπiei of the second aspect, dissolution occurs to give the desned pharmaceutical composition.

In that instance, the imaging agent, plus other optional excipients as described above, may be provided as a lyophilised powdei in a suitable vial or contamei. The agent is then designed to be iieconstituted with the desned biocompatible camel to the pharmaceutical composition in a stenle, apyiogene fomi which is ieady for mammalian admimstiation A piefeπed stenle, solid fomi of the imaging agent is a lyophilised solid The stenle, solid fomi is piefeiably supplied in a pharmaceutical giade contamei, as descibed for the pharmaceutical composition (above) When the kit is lyophilised, the foimulation may optionally compiπse a cryoptotectant chosen from a sacchaπde, piefeiably niannitol, maltose or tiicme

In a fifth aspect, the present invention provides a method of //; vivo optical imaging of the mammalian body which compises use of either the imaging agent of the first aspect of the pharmaceutical composition of the second aspect to obtain images of sites of cMet over-expression or localisation //; vivo

30
The term "optical imaging" has the same meaning as for the first aspect (above). The mammalian body of the fifth aspect is preferably used to obtain the optimal excitation wavelength. A preferably embodiment comprises the steps as follows:

In the method of the fifth aspect, the imaging agent is administered to said mammalian body. By "previously administered" is meant that the step involving the clinician, wherein the imaging agent is given to the patient e.g. as an intravenous injection, has already been earned out prior to imaging. This embodiment includes the use of the imaging agent of the first embodiment for the manufacture of a diagnostic agent for optical imaging in vivo of disease states of the mammalian body wherein c-Met is implicated.

A preferably optical imaging method of the fifth aspect is Fluorescence Reflectance Imaging (FRI). In FRI, the imaging agent of the present invention is administered to a subject to be diagnosed, and subsequently a tissue surface of the subject is illuminated with an excitation light - usually continuous wave (CVV) excitation. The light excites the dye (Bzπ V) Fluorescence from the imaging agent, which is generated by the excitation light, is detected using a fluorescence detector. The emitting light is preferably filtered to separate out the fluorescence component (solely or partially). An image is formed from the fluorescent light. Usually minimal processing is performed (no processing to compute optical parameters such as lifetime, quantum yield etc.) and the image maps the fluorescence intensity. The imaging agent is designed to concentrate in the disease area, producing higher fluorescence intensity. Thus the disease area produces positive contrast in a fluorescence intensity image. The image is preferably obtained using a CCD camera on a chip, such that real-time imaging is possible.

The wavelength of excitation varies depending on the type of dye used. The apparatus for generating the excitation light may be a conventional excitation light source such as: a laser (e.g., ion laser, dye laser or semiconductor laser), halogen light source or xenon light source. Various optical filters may optionally be used to obtain the optimal excitation wavelength.

A preferably FRI method comprises the steps as follows:
(l) a tissue suiface of inteiest within the mammalian body is illuminated with
an excitation light,
(n) fluoiescence from the imaging agent, which is geneiated by excitation of
the dye (BzpM), is detected using a fluoiescence detectoi,
(in) the light detected by the fluoiescence detectoi is optionally filteied to
sepaieate out the fluoiescence component,
(iv) an image of said tissue suiface of mteiest is formed from the fluorescent
light of steps (n) 01 (in)
In step (i), the excitation light is piefeiably continuous wave (CW) m natuie In step
(in), the light detected is piefeiably filteied An especially piefeiicd FRI method is
fluoiescence endoscopy
An alternative imaging method of the fifth aspect uses FDPM (fiequency-domam
photon migiation) This has advantages ovei continuous-wave (CW) methods wheic
gieatei depth of detection of the imaging agent within tissue is impoitant [Sevick-
Muiaca et al, Cuπ Opin Chem Biol , 6, 642 650 (2002)] Foi such fiequency/time
domain imaging, it is advantageous if the BzpM has fluoiescent pioperties which can
be modulated depending on the tissue depth of the lesion to be imaged, and the type of
instrumentation employed
The FDPM method is as follows
(a) exposing light-scatteieing biological tissue of said mammalian body having
a heteiogeneous composition to light fiom a light souice with a pie-
determmed time vaiying intensity to excite the imaging agent, the tissue
multiply-scatteieing the excitation light,
(b) detecting a multiply-scatteied light emission fiom the tissue in iesponse to
said exposing,
(c) quantifying a fluoiescence chaiacteiistic throughout the tissue fiom the
emission by establishing a numbei of values with a piocessoi, the values each
coresponding to a level of the fluoiescence chaiacteiistic at a diferente
position within the tissue, the level of the fluoiescence chaiacteiistic vaiying
with heteiogeneous composition of the tissue, and
(d) geneiatmg an image of the tissue by mapping the heteiogeneous
composition of the tissue in accoidance with the values of step (c)
The fluorescence characteristic of step (c) piecibly corresponds to uptake of the imaging agent and piecibly further compiises mapping a number of quantities corresponding to adsorption and scatteiing coefficients of the tissue before administration of the imaging agent. The fluorescence characteristic of step (c) piecibly corresponds to at least one of fluorescence lifetime, fluorescence quantum efficiency, fluorescence yield and imaging agent uptake. The fluorescence characteristic is piecibly independent of the intensity of the emission and independent of imaging agent concentration.

The quantifying of step (c) piecibly compiises (i) establishing an estimate of the values, (ii) determining a calculated emission as a function of the estimate, (iii) compaing the calculated emission to the emission of said detecting to determine an enoi, (iv) piovidng a modified estimate of the fluorescence characteristic as a function of the enoi. The quantifying piecibly compiises determining the values from a mathematical relationship modelling multiple height-scatterig behaviou of the tissue. The method of the fust option piecibly further compiises monitoring a metabolic pioeity of the tissue \( M \) by detecting variation of said fluorescence characteristic.

The optical imaging of the fifth aspect is piecibly used to help facilitate the management of coloectal cancel (CRC) By the term "management of CRC" is meant use m the detection, staging, diagnosis, monitoring of disease progression of the monitoring of treatment. Further details of suitable optical imaging methods have been liiewed by Sevick-Muiaca et al. [Cuπ Opin Chem Biol, 6, 642-650 (2002)]

In a sixth aspect, the piresent invention provides a method of detection, staging, diagnosis, monitoring of disease progression of the monitoring of treatment of coloectal cancel (CRC) of the mammalian body which compiises the \( \text{in vivo} \) optical imaging method of the fifth aspect.

The invention is illustiated by the non-limiting Examples detailed below. Example 1 provides the synthesis of a biological targeting peptide (Peptide 1), which binds to cMct. Example 2 provides methods of conjugating Bzp \(^M\) dyes of the invention to
peptides, in particular Peptide 1 Example 3 provides data demonstrating that the peptide conjugates of Peptide 1 of the invention tetram affinity for cMet, i.e. that the conjugated dye does not interfere with the biological binding and selectivity. Appropriate low binding to human serum albumin and high stability in plasma was demonstrated. Example 4 shows that the peptide conjugates of the invention exhibit useful turnover background ratios in an animal model of colorectal cancer. Example 5 describes the use of predictive software for the dyes of the invention, and demonstrates that the dyes of the invention lack potentially dangerous metabolites. Example 6 describes the toxicity testing of Compound 6, showing that the anticipated clinical dose was well tolerated and without any drug substance related adverse effects.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Structure of Benzopyrylium dyes of the Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula</td>
<td>DY-630</td>
</tr>
<tr>
<td>R&lt;sup&gt;17&lt;/sup&gt;</td>
<td>Et</td>
</tr>
<tr>
<td>R&lt;sup&gt;18&lt;/sup&gt;</td>
<td>Et</td>
</tr>
<tr>
<td>R&lt;sup&gt;19&lt;/sup&gt;</td>
<td>Bu&lt;sup&gt;'&lt;/sup&gt;</td>
</tr>
<tr>
<td>R&lt;sup&gt;20&lt;/sup&gt;</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>R&lt;sup&gt;21&lt;/sup&gt;</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>R&lt;sup&gt;22&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>E&lt;sup&gt;3&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>E&lt;sup&gt;4&lt;/sup&gt;</td>
<td>-</td>
</tr>
</tbody>
</table>

where R<sup>d</sup> is -(CH<sub>3</sub>)<sub>SO</sub>H, R<sup>6</sup> is -(CH<sub>3</sub>)<sub>2</sub>CO-H and R<sup>1</sup> is -(CH<sub>3</sub>)<sub>2</sub>CO-H

DY-752 has the same rings and substituent pattern as DY-652, but has a pentamethylene linkage (i.e. w = 2 and R<sup>+</sup> = H) in place of the tetramethylene linkage of DY-652.

Abbreviations
Conventional 3-letter and single letter amino acid abbreviations are used.

| Acm | Actamidomethyl |
| ACN | Acetonitrile |
| Boc | t-Butyloxycaibonyl |
| DMF | N,N-Dimethylformamide |
| DMSO | Dimethylsulfoxide |
Example 1: Synthesis of Peptide 1.
A 26-mei bicyclic peptide having 2 Cys-Cys bonds (Cys4-16 and 6-14) having the following sequence was used:

Ac-Ala-Gly-Sei-Cys-Tyi-Cys(Acm)-Sei(tBu)-Gly-Pio-Pio-Aig-Phe-Glu-Cys(Tit)-Cys(tBu)-Cys(Acm)-Tip(Boc)-Cys(Tit)-Tyi(tBu)-

Step (a) Synthesis of protected linear pieceuisoi of Peptide 1

The pieceuisoi hneai peptide has the sequence

Ac-Ala-Gly-Sei-Cys(tBu)-Cys(Tit)-Tyi(tBu)-Cys(Acm)-Sei(tBu)-Gly-Pio-Pio-Aig-Phe-Glu-OtBu-Cys(Acm)-Tip(Boc)-Cys(Tit)-Tyi(tBu)-
Glu(OtBu)-Thr(ψ^Mc-pio)-Glu(OtBu)-Gly-Thi(Bii)-Gly-Gly-Lys(Boc)-PolyMc
was assembled on an Applied Biosystems 433A peptide synthesizer using Fmoc
chemistry starting with 0.1 mmol Rink Amide Novagel resin. An excess of 1 mmol
pie-acti\^ated amino acids (using HBTU) was applied in the coupling steps Glu-Thi
pseudopiolme (Novabiochem 05-20-1 122) was mcopoiated in the sequence. The
resin was transfe πed to a nittogen bubble apparatus and treated with a solution of
acetic anhydride (1 mmol) and NMM (1 mmol) dissolved in DCM (5 mL) for 60 m
The anhydride solution was removed by filtration and the residue washed with DCM
and dried under a stream of nitrogen.

The simultaneous removal of the side-chain protectmg groups and cleavage of the
peptide from the resin was earned out in TFA (10 mL) containing 2.5 % TIS, 2.5 % A-
thiocesol and 2.5 % water for 2 houis and 30 m. The resin was removed by
filtration. TFA removed // vacuo and diethyl ether added to the residue. The foimed
piecipitate was washed with diethyl ether and aii-d πed affoiding 264 mg of ciude
peptide.

Purification by piepaiative HPLC (gadient 20-30 % B ovei 40 m where A =
H2O/0 1 % TFA and B = ACN/0 1 % TFA, flow rate 10 mL/min, column
Phenomenex Luna 5µ C18 (2) 250 x 2120 mm, detection UV 214 nm, product
retention time 30 min) of the piude peptide afforded 100 mg of piude. Peptide 1 lineai
pieciusoil The piude product was analysed by analytical HPLC (gadient 10-40 % B
ovei 10 mL/min where A = H2CVO 1 % TFA and B = ACN/0 1 % TFA, flow rate 0 3
mL/min, column Phenomenex Luna 3µ C18 (2) 50 x 2 mm, detection UV 214 nm,
product retention time 654 nm). Fuiihei product chaiaetisation was earned out
by electrospray mass spectrometry (MHT 2+ calculated 1464 6, M H2O+ found
1465 1.

Step (b) Foiimation of Cys4-16 disulfide bridge

Cys4-16, Ac-Ala-Gly-Sei-Cys-Tyi-Cys(Acm)-Ser-Gly-Pio-Pio-Aig-Phe-Glu-
CyS(Acm)-Ti-P-Cys-Tyi-Glu-Thi-Gly-Gly-Thi-Gly-Gly-GlyLyS-NH

The lineai pieciusoil from step (a) (100 mg) was dissolved in 5 % DMSO/water (200
mL) and the solution adjusted to pH 6 using ammonia. The reaction mixture was
stueed for 5 days. The solution was then adjusted to pH 2 using TFA and most of the
solvent removed by evaporation *in vacuo* The residue (40 nL) was injected in
positions onto a preparative HPLC column for product purification.

Purification by preparative HPLC (gradient 0% B for 10 mm, then 0-40% B over 40
mm where \( A = H_2O/0 \% \) TFA and \( B = ACN/0 \% \) TFA, flow rate 10 nL/min, column Phenomenex Luna 5µ C18 (2) 250 x 21 mm, detection UV 214 nm, product retention time 44 mm) of the residue afforded 72 mg of pure Peptide 1
monocyclic product The pure product (as a mixture of isomers P1 to P3) was
analysed by analytical HPLC (gradient 10-40% B over 10 mm where \( A = H_2O/0 \% \) TFA and \( B = ACN/0 \% \) TFA, flow rate 0.3 mL/min, column Phenomenex Luna 3µ C18 (2) 50 x 2 mm, detection UV 214 nm, product retention time 5.37 mm (P1), 5.61 mm (P2), 6.05 mm (P3)) Further product characterisation was carried out using electrospray mass spectrometry (MH\(^2+\) calculated 1463.6, MH\(^2+\) found 1464.1 (P1), 1464.4 (P2), 1464.3 (P3)

Step (c) Formation of Cys6-14 disulfide bridge (Peptide 1)
The monocyclic product from step (b) (72 mg) was dissolved in 75% AcOH/water
(72 mL) under a blanket of nitrogen 1M HCl (7.2 mL) and 0.05 M 1,1,1-trifluoroethanol (48 mL) were added in that order and the mixture stirred for 45 min 1M ascorbic acid (1
mL) was added giving a colourless mixture Most of the solvents were evaporated *in vacuo* and the residue (18 mL) diluted with water/0% TFA (4 mL) and the product purified using preparative HPLC.

Purification by preparative HPLC (gradient 0% B for 10 mm, then 20-30% B over 40 mm where \( A = H_2O/0 \% \) TFA and \( B = ACN/0 \% \) TFA, flow rate 10 mL/mm, column Phenomenex Luna 5µ C18 (2) 250 x 21 mm, detection UV 214 nm, product retention time 43-53 min) of the residue afforded 52 mg of pure Peptide 1
The pure product was analysed by analytical HPLC (gradient 10-40% B over 10 mm where \( A = H_2O/0 \% \) TFA and \( B = ACN/0 \% \) TFA, flow rate 0.3 mL/mm, column Phenomenex Luna 3µ C18 (2) 50 x 2 mm, detection UV 214 nm, product retention time 6.54 mm) Further product characterisation was carried out using electrospray mass spectrometry (MH\(^2+\) calculated 1391.5, MH\(^2+\) found 1392.5)
Example 2: Synthesis of Peptide Conjugates of Benzopyry Hum DΛes.

General conjugation method
To a solution of Peptide 1 (from Example 1, 4 mg, 14 µmol) in DMF (0.5 mL) was added a solution of BzpM NHS ester (1 mg, 1 µmol) and sym-collidine (8 µL, 60 µmol) in DMF (0.5 mL) The reaction mixture was heated (microwave assisted) at 60 °C for 1 h, then at RT overnight The reaction mixture was then diluted with 20% ACN/water/0.1% TFA (7 mL) and the product purified using preparative HPLC.

Purification and characterisation
Purification by preparative HPLC (gradient 20-40% B over 40 mm where A = H2O/0.1% TFA and B = ACN/0.1% TFA, flow rate 10 mL/min, column Phenomenex Luna 5µ C18 (2) 250 x 21.2 mm, detection UV 214 nm) of the crude peptide afforded pure [Peptide I]-BzpM conjugate The purified product was analysed by analytical HPLC (gradient 10-40% B over 5 mm where A = H2O/0.1% TFA and B = ACN/0.1% TFA, flow rate 0.6 mL/min, column Phenomenex Luna 3µ C18 (2) 20 x 2 mm, detection UV 214 nm) Further product characterisation was carried out using electrospray mass spectrometry The compounds piepared are given in Table 3.

Table 3 Peptide-dye conjugates of Peptide 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>BzpM</th>
<th>Synthesis yield</th>
<th>MS found (MS theoretical)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DY-630</td>
<td>2.1 mg (44%)</td>
<td>1700 7 (MH2+ 1699 7)</td>
</tr>
<tr>
<td>2</td>
<td>DY-631</td>
<td>2.5 mg (60%)</td>
<td>1740 2 (MH2+ 1739 7)</td>
</tr>
<tr>
<td>3</td>
<td>DY-633</td>
<td>2.5 mg (60%)</td>
<td>1747 4 (MH2+ 1746 7)</td>
</tr>
<tr>
<td>4</td>
<td>DY-650</td>
<td>3.1 mg (69%)</td>
<td>1726 1 (MH2+ 1725 7)</td>
</tr>
<tr>
<td>5</td>
<td>DY-651</td>
<td>3.0 mg (77%)</td>
<td>1766 4 (MH2+ 1765 7)</td>
</tr>
<tr>
<td>6</td>
<td>DY-652</td>
<td>3.3 mg (91%)</td>
<td>1813 5 (MH2+ 1812 7)</td>
</tr>
<tr>
<td>7</td>
<td>DY-752</td>
<td>1.8 mg (45%)</td>
<td>1825 9 (MH2+ 1825 7)</td>
</tr>
</tbody>
</table>

Example 3: In Vitro Fluorescence polarisation assay.
Fluorescence polansation assay was used to examine the affinity binding of the imaging agent towards the cMet target as well as the binding piopeities ielated to plasma proteins The principle of the fluorescence polansation method can biiefly be described as follows

Monochromatic light passes through a horizontal polanzmg filei and excites fluoscent molecules in the sample Only those molecules that oriented piopeily in
the vertically polarized plane adsorb light, become excited, and subsequently emit light. The emitted light is measured in both horizontal and vertical planes. The anisotrop} value \(A\), is the ratio between the light intensities following the equation

\[
A = \frac{\text{Intensity with horizontal polarizer} - \text{Intensity with vertical polarizer}}{\text{Intensity with horizontal polarizer} + 2 \times \text{Intensity with vertical polarizer}}
\]

The fluorescence anisotropy measurements were performed in 384-well microplates in a volume of 10 \(\mu\)L in binding buffer (PBS, 0.01\%Tween-20, pH 7.5) using a Tecan Safire fluorescence polarisation plate reader (Tecan, US) at Ex 635/Em 678 nm. The concentration of dye-labelled peptide was held constant (5nM) and the concentration of the human c-Met/Fc chimera (R&D Systems) was varied from 0-250 nM. Binding mixtures were equilibrated in the microplate for 10 min at 30\(^\circ\)C. The observed change in anisotropy was fit to the equation

\[
r_{\text{obs}} = r_{\text{free}} \times \left( \frac{[K_d \cdot c\text{Met TP}] \cdot v(K_d + c\text{Met TP}) - 4 \cdot c\text{Met TP}}{c\text{Met TP} \cdot \gamma} \right)
\]

where \(r_{\text{obs}}\) is the observed anisotropy, \(r_{\text{free}}\) is the anisotropy of the free peptide, \(K_d\) is the dissociation constant, \(c\text{Met}\) is the total c-Met concentration, and \(P\) is the total dye-labelled peptide concentration. The equation assumes that the synthetic peptide and the receptor form a reversible complex in solution with a 1:1 stoichiometry. Data fitting was done via nonlinear regression using SigmaPlot software to obtain the \(K_d\) value (one-site binding).

Compounds 1 to 6 were tested for binding towards human c-Met (Fc chimera). The results showed a \(K_d\) of nM for the binding of all compounds tested to human c-Met (see Table 4).

The change of the polarization value was used to assess the binding of the Compound to human serum albumin as a low change of polarisation value is associated to low binding being appropriate for \textit{in-vivo} use. The plasma protein binding (PPB) was confirmed with Biacore measurements. The stability of the imaging agent in plasma was confirmed by measuring the amount of the Compound left after incubation in mouse plasma for 2 hours at 37\(^\circ\)C.
Table 4: *vitro* properties of Compounds 1-6

<table>
<thead>
<tr>
<th>Compound</th>
<th>Affinity (Kd, nM)</th>
<th>PPB (% change in polarization value)</th>
<th>Binding human serum albumin (Biacore)</th>
<th>Mouse plasma stability (2h, 37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.2</td>
<td>36</td>
<td>Very high</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>33</td>
<td>Very low</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>27</td>
<td>Low</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>4</td>
<td>3.2</td>
<td>55</td>
<td>Very high</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>5</td>
<td>2.2</td>
<td>49</td>
<td>Medium</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>6</td>
<td>0.9</td>
<td>46</td>
<td>Very low</td>
<td>&gt;95%</td>
</tr>
</tbody>
</table>

**Example 4: *Vivo* testing of Compounds 2 to 6.**

(a) Animal Model

Female BALB/c/A nude (Bom) mice were used in the study. The use of the animals was approved by the local ethics committee. BALB/c/A nude mice is an inbred immunocompromised mouse strain with a high take rate for human tumours as compared to other nude mice strains. The mice were 8 weeks old upon arrival and with a body weight of approx. 20 gams at the start of the study. The animals were housed in individually ventilated cages (IVC, Scanbui BK) with HEPA filtered air. The animals had ad libitum access to "Rat and Mouse ni 3 Bleeding" diet (Scanbui BK) and tap water acidified by addition of HCl to a molai concentration of 1 nM (pH 3.0).

The colon cancer cell HT-29 is derived from human colon carcinomas and is reported to express c-Met according to Zeng et al [Clm Exp Metastasis, 21, 409-417 (2004)]. The cell line was proven to be tumorigenic when inoculated subcutaneously into nude mice [Flatmaik et al, Em J Cancel 40,1593-1598 (2004)].

HT-29 cells were grown in McCoy's 5a medium (Sigma # M8403) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Stocks were made at passage number four (P4) and frozen down for storage in liquid nitrogen at 10^7 cells/vial in the respective culture media containing 5% DMSO. On the day of the transplantation, the cells were thawed quickly in 37°C water bath (approx. 2 mm),
washed and resuspended in PBS/2% serum (centrifugation at 1200 rpm for 10 mm)

Though mixing of cells in the vials was ensured every time the cells were aspirated into the dosing syringe. A volume of 0.1 ml of cell suspension was injected s.c. at the shoulder and at the back using a fine boie needle (25 G). The animals were then intramuscularly to the cages and the tumours were allowed to grow for 13-17 days. The animals were allowed an acclimatisation period of at least 5 days before the inoculation procedure.

(b) Procedure

All test substances were reconstituted with PBS from freeze-dried powder. A small stack of white papers was imaged to obtain a flat field image which was used to collect for illumination inhomogeneities. For immobilisation during the optical imaging procedure, the animals were anaesthetised in a coaxial open mask to light surgical level anaesthesia with Isoflurane (typically 1.3-2%), using oxygen as the carrier gas. A small piece of skin (3-5 mm) was removed over parts of the tumours and adjacent muscle using a surgical scalpel and fine scissors while the animal was anaesthetised. This was done to measure the signal from tumour and muscle without interference from the overlying skin tissue. The wound was covered by applying a liquid, non-fluorescent bandage spay (3M, MN, USA).

The injection and body temperature of the animal was monitored with a BioVet system (m2m Imaging Coip, NJ, USA) using a pneumatic sensor underneath the animal and a rectal temperature probe. The BioVet system also supplied external heating using a heating mat set to 40ºC to sustain normal body temperature for the duration of the imaging procedure (2 hours). A Vcnflon catheter was placed in the tail vein for contrast agent administration. Each animal was given one contrast agent injection. The injected volume was 0.1 ml of test compound followed immediately by a 0.2 ml saline flush. Fluorescence images were acquired just prior to injection and then every 30 seconds for 2 hours.

(c) Imaging

Imaging was performed though a clinical laparoscope adapted to use a light source to excite the ieporite and a filtering system to extract the fluorescence component. A 635 nm laser was used for excitation of the ieporite molecule. A Hamamatsu ORCA ERG CCD camera was used as the detector. The camera was operated in 2x2 binning.
mode with 0 gam Standard exposure time for colon imaging was 4s The intensity distribution in the image was collected for illumination inhomogeneities though system calibration data A target to background ratio was computed from legions of metastases placed over the exposed tumours and nominal muscle background.

(d) Results

The test compounds had the following average tumour/muscle ratios (Table 5)

Table 5: Tumour/muscle ratios of Compounds 2 to 6

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average tumour:muscle ratio (2 hours p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.40</td>
</tr>
<tr>
<td>3</td>
<td>1.67</td>
</tr>
<tr>
<td>4</td>
<td>1.52</td>
</tr>
<tr>
<td>5</td>
<td>1.22</td>
</tr>
<tr>
<td>6</td>
<td>1.57</td>
</tr>
</tbody>
</table>

Example 5: Metabolism and Toxicity Prediction.

The software tools Deiek and Meteo were obtained from Lhasa Ltd (22-23 Blenheim Terrace, Leeds LS2 9HD, UK) Deiek is used for predicting toxicity of new chemical entities based on known structure-dependent toxicity Similarly, Meteo predicts likely metabolites of novel chemicals Both tools are based on published and unpublished (but verified) data for chemical compounds The chemical structure of dye DY-652 was input No potentially dangerous metabolites in vitro were predicted.


A limited acute dose toxicity study was conducted to investigate the tolerance of Compound 6 at 100 times the preclinical imaging dose (50 nmol/kg body weight) The compound was injected intravenously in male rats, and the animals were sacrificed at 1, 14, 21 and 28 days post injection (p.i.) At necropsy, the major organs were inspected for gross pathology, and the kidneys were taken into neutral buffered formalin for subsequent histomorphological evaluation A weak blue colouration of the skin and a modelate blue colouration of the mine were observed immediately after
injection, which disappeared within 1 day p.i. At necropsy, the kidneys were diffusely gien on day 1 p.i. Light microscopy showed no Compound 6 related findings in the kidneys. The other minor changes seen were incidental and common in young adult laboratory rats. Strong fluorescence staining of blood vessels in the kidney was observed on day 1 p.i. The staining was reduced by day 14 p.i. and was not discernible from control on day 21 p.i.

No evidence of degeneration, necrosis or inflammation was noted in any of the treated animals, suggesting that the nephrotoxicity of the compound is low. It was concluded that a single intravenous administration of Compound 6 to male rats at 100 times the anticipated clinical dose was well tolerated and without any drug substance related adverse effects.
CLAIMS.

1. An imaging agent which comprises a conjugate of Formula I:

\[
\text{Z}^1\text{-}[c\text{MBP}]-\text{Z}^2
\]

\[
\text{(L)}_n[\text{Bz}^m]\]

where:
\(Z^1\) is attached to the N-terminus of cMBP, and is H or M\(^{10}\);
\(Z^2\) is attached to the C-terminus of cMBP and is OH, OB\(^C\), or M\(^{10}\),

where \(B^c\) is a biocompatible cation;

cMBP is a cMet binding cyclic peptide of 17 to 30 amino acids which comprises the amino acid sequence (SEQ-I):

Cys'-X'-Cys'-X^Gly-Pro-Pro-X'-Phe-Glu-Cys^Trp-Cys^Tyr-X'-X'-X

wherein \(X^1\) is Asn, His or Tyr;
\(X^2\) is Gly, Ser, Thr or Asn;
\(X^3\) is Thr or Arg;
\(X^4\) is Ala, Asp, Glu, Gly or Ser;
\(X^5\) is Ser or Thr;
\(X^6\) is Asp or Glu;

and Cys\(^{a-d}\) are each cysteine residues such that residues a and b as well as c and d are cyclised to form two separate disulfide bonds;

M\(^{10}\) is a metabolism inhibiting group which is a biocompatible group which inhibits or suppresses \textit{in vivo} metabolism of the peptide;

\(L\) is a synthetic linker group of formula \(-(A)_m\) wherein each A is independently -CR\(_2^+\), -CR=CR\(^-\), -C=C\(^-\), -CR\(_2^2\)CO\(_2^+\), -CO\(_2^2\)CR\(_2^+\), -NRCO\(^-\), -CONR\(^-\), -NR(C=O)NR\(^-\), -NR(C=S)NR\(^-\), -SO\(_2^2\)NR\(^-\), -NRSO\(_2^2\), -CR\(_2^2\)OCR\(_2^+\), -CR\(_2^2\)SCR\(_2^+\), -CR\(_2^2\)NRCR\(_2^+\), a \(C_{4-8}\) cycloalkylene group, a \(C_{5-12}\) arylene group, or a \(C_{3-12}\) heteroarylene group, an amino acid, a sugar or a monodisperse polyethylene glycol (PEG) building.
block,
each R is independently chosen from H, C₄ alkyl, C₄ alkenyl, C₄ alkynyl,
C₄ alkoxyalkyl or C₄ hydroxyalkyl,
m is an integer of value 1 to 20,
n is an integer of value 0 or 1,
B/p M is a benzopyrylium dye of Formula II

where

Y¹ is a group of Formula Yᵃ₀¹ Yᵇ

R¹-R⁴ and R⁹-Rᵖ aie independently selected from H, -SO₃M¹, Hal, Rᵃ₀¹ C₃₆ aiyl, wheie each M¹ is independently H or B⁰,
R⁵ is H, C₄ alkyl, C₃₆ aiylsulfoniy, Cl, or R⁵ toogether with one of R⁶, R⁶, and R⁶ may optionally form a 5-01 6- membeied unsaturated aliphatic, unsaturated heteroaliphatic 01 aromatic ring,
R⁶ and R⁶ aie independently Rᵃ gioups,
R⁷ and R⁸ aie independently C₄ alkyl, C₄ sulfoalkyl 01 C₆ hydioxyalkyl 01 optionally together with one 01 both of R⁹ and 01 R¹⁰ may form a 5-01 6- membeied N containing heterocyclic 01 heteroaiyl πng,
X is -CR 01 Rᵖ-, -O-, -S-, -Se-, -NR¹⁶- 01 -CH=CH-, wheie R¹⁴ to R¹⁶ aie independently Rᵃ gioups,
Rᵃ is C₄ alkyl, C₄ sulfoalkyl, C₆ caiboxyalkyl 01 C₆ hydroxyalkyl,
w is 1 0 1 2,
J is a biocompatible anion,
with the proviso that Bzp\textsuperscript{M} comprises at least one sulfonic acid substituent
chosen from the R\textsuperscript{1} to R\textsuperscript{16} groups

2 The imaging agent of Claim 1, where in addition to SEQ-I, the cMBP further
comprises an Asp or Glu residue within 4 amino acid residues of either the C- or N-
cMBP peptide terminus, and (L)\textsubscript{1}[Bzp\textsuperscript{M}] is functionalised with an amine group which
is conjugated to the carboxyl side chain of said Asp or Glu residue to give an amide
bond

3 The imaging agent of either of Claim 1 or Claim 2, wherein in addition to SEQ-
1, the cMBP comprises a Lys residue within 4 amino acid residues of either the C- or N-
cMBP peptide terminus, and (L)\textsubscript{1}[Bzp\textsuperscript{M}] is functionalised with a carboxyl group
which is conjugated to the epsilon amine side chain of said Lys residue to give an
amide bond

4 The imaging agent of any one of Claims 1 to 3, wherein cMBP comprises the
amino acid sequence of either SEQ-2 or SEQ-3

\[
\begin{align*}
\text{Ser-Cys}^a & \cdot \text{X}^1 \cdot \text{Cys}^c \cdot X^2 \cdot \text{Gly-PiO-Pico-X'Phe-Glu-Cys}^d \\
\text{Trp-Cys}^b & \cdot \text{Tyr-X}^4 \cdot X' \cdot X^6
\end{align*}
\] (SEQ-2),

\[
\begin{align*}
\text{Ala-Gly-Sei-Cys}^a \cdot \text{X}^1 \cdot \text{Cys}^l \cdot X^2 \cdot \text{Gly-Pio-Pro-X}^l \cdot \text{Phe-Glu-Cys}^d \\
\text{Tφ-Cys}^b \cdot \text{Tyi-Glu-Thi-Glu-Gly-Thr-Gly-Gly-Gly-Lys}
\end{align*}
\] (SEQ-3).

5 The imaging agent of any one of Claims 1 to 4, wherein X\textsuperscript{3} is Aig

6 The imaging agent of any one of Claims 1 to 5, wherein in addition to SEQ-I,
SEQ-2 or SEQ-3, cMBP further comprises at either the N- or C- terminus a linker
peptide which is chosen from -Gly-Gly-Gly-Lys- (SEQ-4), -Gly-Ser-Gly-Lys- (SEQ-
5) or -Gly-Ser-Gly-Ser-Lys- (SEQ-6)

7 The imaging agent of Claim 6, where cMBP has the amino acid sequence
(SEQ-7)

\[
\begin{align*}
\text{Ala-Gly-Ser-Cys}^a & \cdot \text{Tyr-Cys}^c \cdot \text{Ser-Gly-PiO-Pro-Phe-Glu-Cys}^d \\
\text{Trp-Cys}^b & \cdot \text{Tyi-Glu-Thi-Glu-Gly-Thr-Gly-Gly-Gly-Lys}
\end{align*}
\]
The imaging agent of any one of Claims 1 to 7, where both $Z^1$ and $Z^2$ are independently $M$.

The imaging agent of Claim 8, where $Z^1$ is acetyl and $Z^2$ is a pnmaiy amide.

The imaging agent of any one of Claims 1 to 9, where $n$ is 0.

The imaging agent of any one of Claims 1 to 10, where $Bzp^M$ is of Formula IIa.

The imaging agent of any one of Claims 1 to 10, where $Bzp^M$ is of Formula III.

The imaging agent of any one of Claims 1 to 12, where $Bzp^M$ comprises 2 to 4 sulfonic acid substituents.

The imaging agent of any one of Claims 1 to 13, where $Bzp^M$ comprises at least one $C_4$ sulfoalkyl substituent.

The imaging agent of any one of Claims 1 to 14, where $Bzp^M$ is of Formula III.
where $Y^1, R^1 - R^4, R^6, R^{14}, R^{15}$ and $J$ are as defined in Claim 1.

16. The imaging agent of Claim 15, which is of Formula IHc, IHd or IHe:

where:

(III)

(IIIc)

(HTd)

(HHe)
M1 is H or B\ wheie B is as defined m Claim 1.
R17 and R18 are independently chosen from C4 alkyl or C4 sulfoalkyl.
R19 is H or C14 alkyl,
R20 is C4 alkyl, C4 sulfoalkyl or C6 carboxyalkyl,
5 R21 is C4 sulfoalkyl or C6 carboxyalkyl,
R27 is C4 alkyl, C4 sulfoalkyl or C6 carboxyalkyl,
E2, E1 and E4 are independently H or C4 alkyl

17 The imaging agent of any one of Claims 1 to 16, wheie cMBP is as defined in
10 Claim 7, Z1 and Z2 are as defined in Claim 9 and BzpM is as defined in any one of
Claims 11 to 16

18 A pharmaceutical composition which comprises the imaging agent of any one
15 of Claims 1 to 17 together with a biocompatible ca\ piei, in a formula suitable for
mammalian administration

19 The pharmaceutical composition of Claim 18, which has a dosage suitable for
a single patient and is provided in a suitable syringe or container

20 A method of piepaiation of the imaging agent of Claims 1 to 17, which
comprises one of steps (1) to (iv)

(1) Reaction of a peptide of formula Z1 [cMBP]-Z2 whence Z1 is H and Z2 is a
M4 with a compound of formula \( j^-{(L)}^n [Bzp^M] \), to give the imaging agent of
Formula I wherein BzpM is conjugated at the Z1 position,
25 (11) Reaction of a peptide of formula Z1 [cMBP]-Z2 whence Z1 = Z2 = M4 and
cMBP comprises an Asp GLu residue within 4 amino acid residues of either the C- or N- cMBP peptide terminus, and all other Asp/Gr residues of the
cMBP peptides are protected, with a compound of formula
\( T^-{(L)}^n [Bzp^M] \), to give the imaging agent of Formula I wherein BzpM is
30 conjugated at said Asp GLu residue of the cMBP peptide.

(111) Reaction of a peptide of formula Z1 [cMBP]-Z2 whence Z1 is M4 and Z2 is a Z2 group in an activated ester and all other Asp/Gr residues of the cMBP
peptides are protected, with a compound of formula
\( T^-{(L)}^n [Bzp^M] \), to give the imaging agent of Formula I wherein BzpM is
conjugated at the \( Z \) position,

(iv) reaction of a peptide of formula \( Z'\)-[cMBP]-Z'\) wherein \( Z_1 = Z_2 = M_1 \) and cMBP comp ses a Lys within 4 amino acid residues of either the C- or N- cMBP peptide tei minus, with a compound of formula \( j'(L)_{n}\)-[Bzp \( M \)], to give

the imaging agent of Formula I wherein Bzp \( M \) is conjugated at a Lys residue of the cMBP peptide,

\[ Z_1, cMBP, Z_2, M_1, L_n \text{ and } Bzp M \text{ are as defined in Claim 1}, \]

\( Z' \) is a Z group of an activated ester,

\( J^1 \) is a activated ester, isothiocyanate or thiocyanate group,

\( J^2 \) is an amine group

21 The method of Claim 20, wherein the reaction of step (iv) is used

22 A kit for the preparation of the pharmaceutical composition of Claim 18 or 19, which comprises the imaging agent of Claims 1 to 17 in sterile, solid form such that upon reconstitution with a sterile supply of the biocompatible carrier as defined in Claim 18 or 19, dissolution occurs to give the desired pharmaceutical composition

23 The kit of Claim 22, wherein sterile, solid form is a lyophilised solid

24 A method of \textit{in vivo} optical imaging of the mammalian body which comprises use of either the imaging agent of Claims 1 to 17 or the pharmaceutical composition of Claims 18 or 19 to obtain images of sites of cMet over-expression or localisation \textit{in vivo}

25 The method of Claim 24, wherein the imaging agent of Claims 1 to 17 or the pharmaceutical composition of Claims 18 or 19 has been previously administered to said mammalian body

26 The method of Claim 25, which comprises the steps of

(i) a tissue surface or tissue within the mammalian body is illuminated with an excitation light,

(ii) fluorescence from the imaging agent, which is generated by excitation of
the BzpM, is detected using a fluorescence detector,
(in) the light detected by the fluorescence detector is optionally filtered to separate out the fluorescence component,
(iv) an image of said tissue surface of interest is formed from the fluorescent light of steps (ii) or (in)

27 The method of Claim 26 where the excitation light of step (i) is continuous wave (CW) in nature.

28 The method of Claim 25, which comprises
(a) exposing light-scattering biologic tissue of said mammalian body having a heterogeneous composition to light from a light source with a predetermined time varying intensity to excite the imaging agent, the tissue multiply-scattering the excitation light,
(b) detecting a multiply-scattered light emission from the tissue in response to said exposing,
(c) quantifying a fluorescence characteristic throughout the tissue from the emission by establishing a number of values with a processor, the values each corresponding to a level of the fluorescence characteristic at a different position within the tissue, the level of the fluorescence characteristic varying with heterogeneous composition of the tissue, and
(d) generating an image of the tissue by mapping the heterogeneous composition of the tissue in accordance with the values of step (c)

29 The method of any one of Claims 24 to 28, where the optical imaging method comprises fluorescence endoscopy

30 The method of any one of Claims 24 to 29, where the in vivo optical imaging is used to assist in the detection, staging, diagnosis, monitoring of disease progression or monitoring of treatment of colorectal cancer (CRC)

31 A method of detection, staging, diagnosis, monitoring of disease progression or monitoring of treatment of colorectal cancer (CRC) of the mammalian body which comprises the in vivo optical imaging method of any one of Claims 24 to 29