Peptide Imaging Agents.

Field of the Invention.

The present invention relates to labelled cMet binding peptides suitable for optical imaging *in vivo*. 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, Her-2, matrix metalloproteinases (MMPs), cyclins, P53, thymidylate synthase, VEGF receptors, EGF receptors, K-ras, adenomatous polyposis coli protein, cathepsin B. uPAR, cMet, mucins and gastrin receptors. Preferred such targets (p.7 lines 11-12) are said to be: cMet, MMP-14, COX-2, beta-catenin and Cathepsin B. The vector of WO 2005/030266 can be: a peptide, peptoid moiety, oligonucleotide, oligosaccharide, lipid-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 or multimeric peptide constructs which bind

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cMet or 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 *in vitro* and *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 radionuclide. 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.

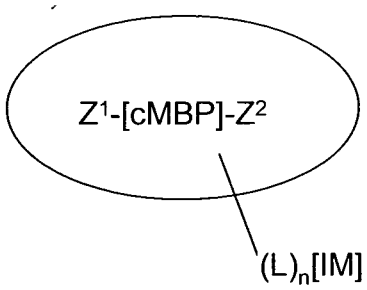
The Present Invention.

The present invention provides imaging agents suitable for *in vivo* optical imaging, which comprise cMet binding cyclic peptides, and an optical reporter imaging moiety suitable for imaging the mammalian body *in vivo* using light of green to near-infrared wavelength 500-1200 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^{IG}). That is an important consideration for *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 *in vivo*.

The present invention teaches that the best way of using cMet binding peptides *in vivo* involves the use of an optical reporter, as opposed to other imaging modalities (eg. nuclear, MRI or ultrasound), and also provides preferred optical imaging reporters. The green to near-infrared region (light of wavelength 500-1200 nm) is preferred, since that region has minimal spectral overlap with endogenous tissues and materials, such as haemoglobin, porphyrins, melanin, and collagen [Licha, Topics Curr.Chem., 222, 1-29 (2002)]. Other important contributors to 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:



(I)

where:

Z^1 is attached to the N-terminus of cMBP, and is H or M^{IG};

Z^2 is attached to the C-terminus of cMBP and is OH, OB^c, or M^{IG},

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-1):

Cys^a-X^1-Cys^c-X^2-Gly-Pro-Pro-X^3-Phe-Glu-Cys^d-Trp-Cys^b-Tyr-X^4-X^5-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^{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^{IG} is a metabolism inhibiting group which is a biocompatible group which inhibits or suppresses *in vivo* metabolism of the cMBP peptide;

L is a synthetic linker group of formula $-(A)_m-$ wherein each A is independently $-CR_2-$, $-CR=CR-$, $-C\equiv C-$, $-CR_2CO_2-$, $-CO_2CR_2-$, $-NRCO-$, $-CONR-$, $-NR(C=O)NR-$, $-NR(C=S)NR-$, $-SO_2NR-$, $-NRSO_2-$, $-CR_2OCR_2-$, $-CR_2SCR_2-$, $-CR_2NRCR_2-$, a C_{4-8} cycloheteroalkylene group, 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 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;

IM is an optical reporter imaging moiety suitable for imaging the mammalian body *in vivo* using light of green to near-infrared wavelength 600-1200 nm.

By the term "imaging agent" is meant a compound suitable for imaging the mammalian body *in vivo.* Preferably, the mammal is a human subject. The imaging may be invasive (eg. intra-operative or endoscopic) or non-invasive. The preferred imaging method is endoscopy. Whilst the conjugate of Formula I is suitable for *in vivo* imaging, it may also have *in vitro* applications (eg. assays quantifying cMet in biological samples or visualisation of cMet in tissue samples). Preferably, the imaging agent is used for *in vivo* imaging.

The Z^1 group substitutes the amine group of the last amino acid residue. Thus, when Z^1 is H, the amino terminus of the cMBP terminates in a free NH_2 group of the last amino acid residue. The Z^2 group substitutes the carbonyl group of the last amino acid residue. Thus, when Z^2 is OH, the carboxy terminus of the cMBP terminates in the free CO_2H group of the last amino acid residue, and when Z^2 is OB^c that terminal carboxy group is ionised as a CO_2B^c group.

By the term "metabolism inhibiting group" (M^{IG}) is meant a biocompatible group which inhibits or suppresses *in vivo* metabolism of the cMBP peptide at either the amino terminus (Z^1) or carboxy 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 $-NH(C=O)R^G$ where the acyl group $-(C=O)R^G$ has R^G chosen from: C_{1-6} alkyl, C_{3-10} aryl groups or comprises a polyethyleneglycol (PEG) building block. Suitable PEG groups are described for the linker group (L), below. Preferred such PEG groups are the biomodifiers of Formula IA or IB. Preferred such amino terminus M^{IG} groups are acetyl, benzyloxycarbonyl or trifluoroacetyl, most preferably acetyl.

Suitable metabolism inhibiting groups for the peptide carboxyl terminus include:

carboxamide, *tert*-butyl ester, benzyl ester, cyclohexyl ester, amino alcohol or a polyethyleneglycol (PEG) building block. A suitable M^{IG} group for the carboxy terminal amino acid residue of the cMBP peptide is where the terminal amine of the amino acid residue is N-alkylated with a C_{1-4} alkyl group, preferably a methyl group. Preferred such M^{IG} groups are carboxamide or PEG, most preferred such groups are carboxamide.

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

The $-(L)_n-$ moiety of Formula I may be attached at any suitable position of the IM. The $-(L)_n-$ moiety either takes the place of an existing substituent of the IM, or is covalently attached to the existing substituent of the IM. The $-(L)_n-$ moiety is preferably attached *via* a carboxyalkyl substituent of the IM.

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 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 proline 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. Preferred biocompatible cations are sodium and potassium, most preferably sodium.

By the term "amino acid" is meant an L- or D-amino acid, amino acid analogue (eg. 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 herein. 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-inverso peptides, thioamides, cycloalkanes or 1,5-disubstituted tetrazoles [see M. Goodman, Biopolymers, 24, 137, (1985)].

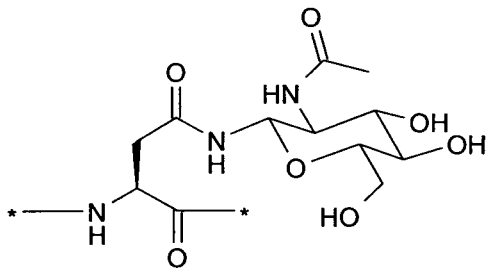
By the term "peptide" is meant a compound comprising two or more amino acids, as defined above, linked by a peptide bond (ie. an amide bond linking the amine of one amino acid to the carboxyl of another). The term "peptide mimetic" or "mimetic" refers to biologically active compounds that mimic the biological activity of a peptide or 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.

By the term "optical reporter imaging moiety" (IM) is meant a fluorescent dye or chromophore which is capable of detection either directly or indirectly in an optical imaging procedure using light of green to near-infrared wavelength (500-1200 nm, preferably 600-1000 nm). Preferably, the IM has fluorescent properties.

It is envisaged that one of the roles of the linker group $-(A)_m-$ of Formula I is to distance the IM from the active site of the cMBP peptide. This is particularly important when the imaging moiety is relatively bulky, so that interaction with the enzyme is not impaired. This can be achieved by a combination of flexibility (eg. 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 aryl spacer which orientate the IM away from the active site. The nature of the linker group can also be used to modify the biodistribution of the imaging agent. Thus, eg. the introduction of ether groups in the linker will help to minimise plasma protein binding. When $-(A)_m-$ comprises a polyethyleneglycol (PEG) building block or a peptide chain of 1 to 10 amino acid residues, the linker group may function to modify the pharmacokinetics and blood clearance rates of the imaging agent *in vivo*. Such "biomodifier" linker 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 biomodifier linker group may also be used to favour a particular route of excretion, eg. *via* the kidneys as opposed to *via* the liver.

By the term "sugar" is meant a mono-, di- or tri- saccharide. Suitable sugars include: glucose, galactose, maltose, mannose, and lactose. Optionally, the sugar may be functionalised to permit facile coupling to amino acids. Thus, eg. a glucosamine derivative of an amino acid can be conjugated to other amino acids *via* peptide bonds. The glucosamine derivative of asparagine (commercially available from NovaBiochem) is one example of this:



Preferred features.

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

Preferred imaging agents of the present invention have both peptide termini protected by M^{IG} groups, ie. preferably both Z^1 and Z^2 are M^{IG}, which will usually be different.

As noted above, either of Z^1/Z^2 may optionally equate to $-(L)_n[IM]$. Having both peptide termini protected in this way is important for *in vivo* imaging applications, since otherwise rapid metabolism would be expected with consequent loss of selective binding affinity for cMet. When both Z^1 and Z^2 are M^{IG}, preferably Z^1 is acetyl and Z^2 is a primary amide. Most preferably, Z^1 is acetyl and Z^2 is a primary amide and the $-(L)_n[IM]$ moiety is attached to the epsilon amine side chain of a lysine residue of cMBP.

Preferred cMBP peptides of the present invention have a K_D for binding of cMet to cMet/HGF complex of less than about 10 nM (based on fluorescence polarisation assay measurements), most preferably in the range 1 to 5 nM, with less than 3nM being the ideal.

The peptide sequence (SEQ-1)

$$Cys^a\text{-}X^1\text{-}Cys^c\text{-}X^2\text{-}Gly\text{-}Pro\text{-}Pro\text{-}X^3\text{-}Phe\text{-}Glu\text{-}Cys^d\text{-}Trp\text{-}Cys^b\text{-}Tyr\text{-}X^4\text{-}X^5\text{-}X^6$$

(SEQ-1)

of the cMBP of Formula I is a 17-mer peptide sequence, which is primarily responsible for the selective binding to cMet. When the cMBP peptide of the present invention comprises more than 17 amino acid residues, the remaining 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 $-(L)_n[IM]$ moiety. Suitable such residues include Asp or Glu residues for conjugation with amine-functionalised –(L)n[IM] groups, or a Lys residue for conjugation with a carboxy- or active ester-functionalised –(L)n[IM] group. The amino acid residues for conjugation of $-(L)_n[IM]$ are suitably located away from the 17-mer binding region of the cMBP peptide (SEQ-1), 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-1 was evaluated with the known amino acid substitutes phenylalanine and napthylalanine. Loss of cMet affinity was, however, found suggesting that the tryptophan residue is important for activity.

It is preferred that the cMBP peptide further comprises a N-terminal serine residue, giving the 18-mer (SEQ-2):

Ser-Cys^a-X^1-Cys^c-X^2-Gly-Pro-Pro-X^3-Phe-Glu-Cys^d-Trp-Cys^b-Tyr-X^4-X^5-X^6. (SEQ-2)

In addition to SEQ-1, or preferably SEQ-2, the cMBP most preferably further comprises either:

(i) an Asp or Glu residue within 4 amino acid residues of either the C- or N-peptide terminus of the cMBP peptide, and -(L)$_n$IM 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;

(ii) a Lys residue within 4 amino acid residues of either the C- or N- peptide terminus of the cMBP peptide, and -(L)$_n$IM is functionalised with a carboxyl group which is conjugated to the epsilon amine side chain of said Lys residue to give an amide bond.

Preferred cMBP peptides comprise the 22-mer amino acid sequence (SEQ-3):

Ala-Gly-Ser-Cys^a-X^1-Cys^c-X^2-Gly-Pro-Pro-X^3-Phe-Glu-Cys^d-Trp-Cys^b-Tyr-X^4-X^5-X^6-Gly-Thr. (SEQ-3)

The cMBP peptides of the present invention preferably have X^3 equal to Arg.

The cMBP peptide preferably further comprises in addition to SEQ-1, SEQ-2 or SEQ-3, 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).

The Lys residue of the linker peptide is a most preferred location for conjugation of the –(L)$_n$[IM] moiety. Especially preferred cMBP peptides comprise SEQ-3 together with the linker peptide of SEQ-4, having the 26-mer amino acid sequence (SEQ-7):

Ala-Gly-Ser-Cys^a-Tyr-Cys^c-Ser-Gly-Pro-Pro-Arg-Phe-Glu-Cys^d-Trp-Cys^b-Tyr-Glu-Thr-Glu-Gly-Thr-Gly-Gly-Gly-Lys. (SEQ-7)

cMBP peptides of SEQ-1, SEQ-2, SEQ-3 and SEQ-7 preferably have Z^1 = Z^2 = M^{IG}, and most preferably have Z^1 = acetyl and Z^2 = primary amide.

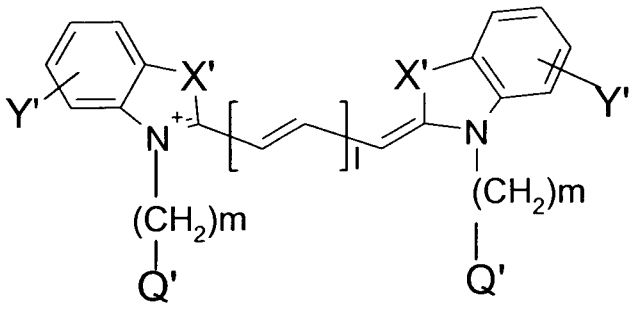
The –(L)$_n$[IM] moiety is suitably attached to either of the Z^1 or Z^2 groups or an amino

acid residue of the cMBP peptide which is different to the cMet binding sequence of SEQ-1. Preferred amino acid residues and sites of conjugation are as described above. When the $-(L)_n[IM]$ moiety is attached to Z^1 or Z^2, it may take the place of Z^1 or Z^2 by conjugation to the N- or C- terminus, and block *in vivo* metabolism in that way.

Preferred IM groups have an extensive delocalized electron system, eg. cyanines, merocyanines, indocyanines, phthalocyanines, naphthalocyanines, triphenylmethines, porphyrins, pyrilium dyes, thiapyrilium dyes, squarylium dyes, croconium dyes, azulenium dyes, indoanilines, benzophenoxazinium dyes, benzothiaphenothiazinium dyes, anthraquinones, napthoquinones, indathrenes, phthaloylacridones, *tris*phenoquinones, azo dyes, intramolecular and intermolecular charge-transfer dyes and dye complexes, tropones, tetrazines, *bis*(dithiolene) complexes, *bis*(benzene-dithiolate) complexes, iodoaniline dyes, *bis*(S,O-dithiolene) complexes. Fluorescent proteins, such as green fluorescent protein (GFP) and modifications of GFP that have different absorption/emission properties are also useful. Complexes of certain rare earth metals (e.g., europium, samarium, terbium or dysprosium) are used in certain contexts, as are fluorescent nanocrystals (quantum dots).

Particular examples of chromophores which may be used include fluorescein, sulforhodamine 101 (Texas Red), rhodamine B. rhodamine 6G, rhodamine 19, indocyanine green, Cy2, Cy3B, Cy3.5, Cy5, Cy5.5, Cy7, Cy7.5, Marina Blue, Pacific Blue, Oregon Green 488, Oregon Green 514, tetramethylrhodamine, and Alexa Fluor 350, Alexa Fluor 430, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 633, Alexa Fluor 647, Alexa Fluor 660, Alexa Fluor 680, Alexa Fluor 700, and Alexa Fluor 750. The cyanine dyes are particularly preferred. Licha *et al* have reviewed dyes and dye conjugates for *in vivo* optical imaging [Topics Curr.Chem., 222, 1-29 (2002); Adv.Drug Deliv.Rev., 57, 1087-1108 (2005)].

Preferred cyanine dyes which are fluorophores are of Formula II:



(II)

wherein:

each X' is independently selected from: $-C(CH_3)_2$, -S-, -O- or

$-C[(CH_2)_aCH_3][(CH_2)_bM]-$, wherein a is an integer of value 0 to 5, b is an integer of value 1 to 5, and M is group G or is selected from SO_3M^1 or H;

each Y' independently represents 1 to 4 groups selected from the group consisting of:

H, $-CH_2NH_2$, $-SO_3M^1$, $-CH_2COOM^1$, -NCS and F, and wherein the Y' groups are placed in any of the positions of the aromatic ring;

Q' is independently selected from the group consisting of: H, SO_3M^1, NH_2, $COOM^1$,

ammonium, ester groups, benzyl and a group G;

M^1 is H or B^c;

l is an integer from 1 to 3;

and m is an integer from 1 to 5;

wherein at least one of X', Y' and Q' comprises a group G;

G is a reactive or functional group suitable for attaching to the cMBP peptide.

The G group reacts with a complementary group of the cMBP peptide forming a

covalent linkage between the cyanine dye fluorophore and the cMBP peptide. G may be a reactive group 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; maleimide; haloacetamide; acid halide; hydrazide; vinylsulphone; dichlorotriazine; phosphoramidite; hydroxyl; amino; sulphydryl; carbonyl; carboxylic acid and thiophosphate. Preferably G is an active ester.

By the term "activated ester" or "active ester" is meant an ester derivative of the associated carboxylic acid which is designed to be a better leaving group, and hence permit more facile reaction with nucleophile, such as amines. Examples of suitable active esters are: N-hydroxysuccinimide (NHS), sulpho-succinimidyl ester, pentafluorophenol, pentafluorothiophenol, *para*-nitrophenol, hydroxybenzotriazole and PyBOP (ie. benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate). Preferred active esters are N-hydroxysuccinimide or pentafluorophenol esters, especially N-hydroxysuccinimide esters.

In a preferred embodiment of Formula II:

each X′ is selected from the group of $-C(CH_3)_2-$ and $-C(CH_3)[(CH_2)_4M]-$,

wherein M is a G group or $-SO_3M^1$;

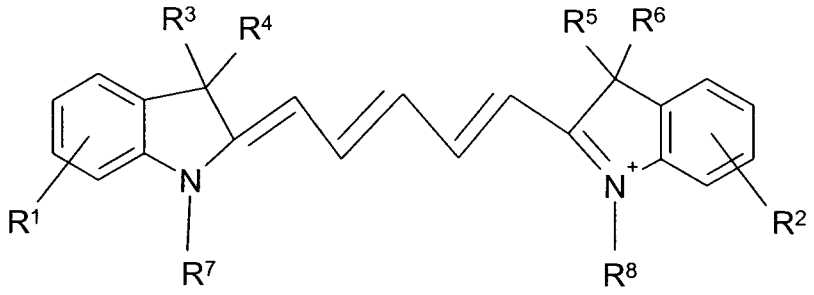
each Y′ represents SO_3M^1, H or 1 to 4 F atoms;

each Q′ is selected from a G group and SO_3M^1;

l is preferably 2 and m is preferably 3, 4 or 5;

wherein when either X′ or Q′ is a G group, it is most preferably a succinimidyl ester.

Particularly preferred cyanine dyes are of Formula III:



(III)

where:

R^1 and R^2 are independently H or SO_3M^1, and at least one of R^1 and R^2 is SO_3M^1, where M^1 is H or B^c;

R^3 and R^4 are independently C_{1-4} alkyl or C_{1-6} carboxyalkyl;

R^5, R^6, R^7 and R^8 are independently R^a groups;

wherein R^a is C_{1-4} alkyl, C_{1-6} carboxyalkyl or $-(CH_2)_kSO_3M^1$, where k is an integer of value 3 or 4;

with the proviso that the cyanine dye has a total of 1 to 4 SO_3M^1 substituents in the R^1, R^2 and R^a groups.

Preferred dyes of Formula III are chosen such that at least one C_{1-6} carboxyalkyl group is present, in order to facilitate conjugation to the cMBP.

Preferred individual dyes of Formula III are summarised in Table 1:

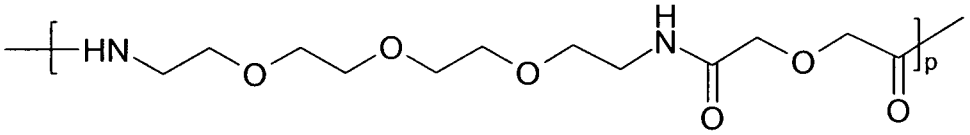
Table 1: chemical structures of individual cyanine dyes.

Table 1	**Dye name**			
	Cy5(1)	**Cy5(2)**	**Cy5****	**Alexa647**
R^1	H	SO_3H	SO_3H	SO_3H
R^2	SO_3H	SO_3H	SO_3H	SO_3H
R^3	CH_3	CH_3	CH_3	R^f
R^4	CH_3	CH_3	CH_3	CH_3
R^5	CH_3	CH_3	CH_3	CH_3
R^6	CH_3	CH_3	$-(CH_2)_4SO_3H$	CH_3
R^7	R^f	R^f	R^f	$-(CH_2)_3SO_3H$
R^8	CH_3	Et	$-(CH_2)_4SO_3H$	$-(CH_2)_3SO_3H$

where $R^f = -(CH_2)_5COOH$.

Especially preferred dyes of Formula II are Cy5** and Alexa647, with Cy5** being the ideal.

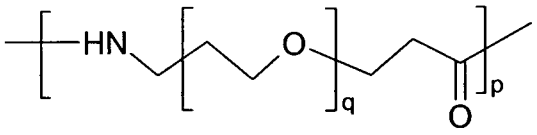
When a synthetic linker group (L) is present, it preferably comprises terminal functional groups which facilitate conjugation to [IM] and Z^1-[cMBP]-Z^2. When L comprises a peptide chain of 1 to 10 amino acid residues, the amino acid residues are preferably chosen from glycine, lysine, arginine, aspartic 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 IA or IB:



(IA)

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

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



(IB)

where p is as defined for Formula IA and q is an integer from 3 to 15.

In Formula IB, 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 L 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 undesirable 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:

M^{IG}-Ala-Gly-Ser-Cys^a-Tyr-Cys^c-Ser-Gly-Pro-Pro-Arg-Phe-Glu-Cys^d-Trp-Cys^b-Tyr-Glu-Thr-Glu-Gly-Thr-Gly-Gly-Gly-Lys-M^{IG}

$(L)_n[IM]$

(IV)

wherein the $(L)_n[IM]$ group is attached to the epsilon amino group of the Lys residue. Preferred imaging agents of Formula IV have M^{IG} (N-terminal Ala) equal to acetyl and M^{IG} (C-terminal Lys) equal to primary amide. In Formula IV, n is preferably zero and IM is preferably a cyanine dye, most preferably a cyanine dye of Formula II. Especially preferred imaging agents of Formula IV have IM = Cy5** or Alexa647, ideally Cy5**.

Peptides of formula Z^1-[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-protecting groups;

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

(iii) removal of the Cys^c and Cys^d thiol-protecting groups and cyclisation to give a second disulphide bond linking Cys^c and Cys^d, which is the desired bicyclic peptide product Z^1-[cMBP]-Z^2.

By the term "protecting group" is meant a group which inhibits or suppresses undesirable chemical reactions, but which is designed to be sufficiently reactive 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 fluorenylmethoxycarbonyl), trifluoroacetyl, allyloxycarbonyl, Dde [i.e. 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl] or Npys (i.e. 3-nitro-2-pyridine sulfenyl). Suitable thiol protecting groups are Trt (Trityl), Acm (acetamidomethyl), *t*-Bu (tert-butyl), *tert*-Butylthio, methoxybenzyl, methylbenzyl or Npys (3-nitro-2-pyridine sulfenyl). The use of further protecting groups are described in 'Protective Groups in Organic Synthesis', Theorodora W. Greene and Peter G. M. Wuts, (John Wiley & Sons, 1991). Preferred amine protecting groups are Boc and Fmoc, most preferably Boc. Preferred amine protecting groups are Trt and Acm.

Examples 1 and 2 provide further specific details. Further details of solid phase peptide synthesis are described in P. Lloyd-Williams, F. Albericio and E. Girald; *Chemical Approaches to the Synthesis of Peptides and Proteins*, CRC Press, 1997. The cMBP peptides are best stored under inert atmosphere and kept in a freezer. When used in solution, it is best to avoid pH above 7 since that risks scrambling of the disulfide bridges.

The imaging agents can be prepared as described in the third aspect (below).

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

The "biocompatible carrier" is a fluid, especially a liquid, in which the imaging agent can be suspended or dissolved, such that the composition is physiologically tolerable, ie. can be administered to the mammalian body without toxicity or undue discomfort. The biocompatible carrier is suitably an injectable carrier 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 or more tonicity-adjusting substances (eg. salts of plasma cations with biocompatible counterions), sugars (e.g. glucose or sucrose), sugar alcohols (eg. sorbitol or mannitol), glycols (eg. glycerol), or other non-ionic polyol materials (eg. polyethyleneglycols, propylene glycols and the like). Preferably the biocompatible carrier is pyrogen-free water for injection or isotonic saline.

The imaging agents and biocompatible carrier are each supplied in suitable vials or vessels which comprise a sealed container which permits maintenance of sterile integrity and/or radioactive safety, plus optionally an inert headspace gas (eg. nitrogen or argon), whilst permitting addition and withdrawal of solutions by syringe or cannula. A preferred such container is a septum-sealed vial, wherein the gas-tight closure is crimped on with an overseal (typically of aluminium). The closure is suitable for single or multiple puncturing with a hypodermic needle (e.g. a crimped-on septum seal closure) whilst maintaining sterile integrity. Such containers have the additional advantage that the closure can withstand vacuum if desired (eg. 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.

Preferred multiple dose containers comprise a single bulk vial (e.g. of 10 to 30 cm^3 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. Pre-filled syringes are designed to contain a single human dose, or "unit dose" and are therefore preferably a 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-organism in the pharmaceutical composition. The antimicrobial preservative may, however, also optionally be used to inhibit the growth of potentially harmful micro-organisms in one or more components of kits used to prepare said composition prior to administration. Suitable antimicrobial preservative(s) include: the parabens, ie. methyl, ethyl, propyl

or butyl paraben or mixtures thereof; benzyl alcohol; phenol; cresol; cetrimide and thiomersal. Preferred antimicrobial preservative(s) are the parabens.

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 tricine, phosphate or TRIS [ie. *tris*(hydroxymethyl)aminomethane], 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 user 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 chloride, and water soluble sugars or sugar alcohols such as sucrose, maltose, mannitol or trehalose.

The pharmaceutical compositions of the second aspect may be prepared under aseptic manufacture (ie. clean room) conditions to give the desired sterile, non-pyrogenic product. It is preferred that the key components, especially the associated reagents plus those parts of the apparatus which come into contact with the imaging agent (eg. vials) are sterile. The components and reagents can be sterilised by methods known in the art, including: sterile filtration, terminal sterilisation 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 prepared from a kit, as described for the fourth aspect below.

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

(i) reaction of a cMBP peptide of formula Z^1-[cMBP]-Z^2 wherein Z^1 is H and Z^2 is a M^{IG} with a compound of formula Y^1-$(L)_n$-[IM], to give the imaging agent of Formula I wherein [IM] is conjugated at the Z^1 position;

(ii) reaction of a cMBP peptide of formula Z^1-[cMBP]-Z^2 wherein $Z^1 = Z^2 = M^{IG}$ and cMBP comprises an Asp or Glu residue within 4 amino acid residues of either the C- or N- cMBP peptide terminus, and all other Asp/Glu residues of the cMBP peptide are protected, with a compound of formula Y^2-$(L)_n$-[IM], to give the imaging agent of Formula I wherein [IM] is conjugated at said Asp or Glu residue of the cMBP peptide;

(iii) reaction of a cMBP peptide of formula Z^1-[cMBP]-Z^3 wherein Z^1 is M^{IG} and Z^3 is a Z^2 group or an activated ester and all other Asp/Glu residues of the cMBP peptide are protected, with a compound of formula Y^2-$(L)_n$-[IM], to give the imaging agent of Formula I wherein [IM] is conjugated at the Z^2 position;

(iv) reaction of a cMBP peptide of formula Z^1-[cMBP]-Z^2 wherein $Z^1 = Z^2 = M^{IG}$ and cMBP comprises a Lys within 4 amino acid residues of either the C- or N- cMBP peptide terminus, with a compound of formula Y^1-$(L)_n$-[IM], to give the imaging agent of Formula I wherein [IM] is conjugated at a Lys residue of the cMBP peptide;

wherein Z^1, cMBP, Z^2, M^{IG}, L, n and IM are as defined in the first aspect (above), and

Z^3 is a Z^2 group or an activated ester;

Y^1 is a carboxylic acid, activated ester, isothiocyanate or thiocyanate group;

Y^2 is an amine group.

The terms "activated ester" or "active ester" and preferred embodiments thereof are as described above. Y^2 is preferably a primary or secondary amine group, most preferably a primary amine group.

The compound Z^1-[cMBP]-Z^2 preferably has both Z^1 and Z^2 equal to M^{IG}. Preferred cMBP peptides and Z^1/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^1-[cMBP]-Z^2 is described in the first embodiment (above). The Z^1-[cMBP]-Z^3 peptide where Z^3 is an active ester can be prepared from Z^1-[cMBP]-Z^2, where Z^2 is OH or a biocompatible cation (B^c), by conventional methods.

Optical reporter dyes (IM) functionalised suitable for conjugation to peptides are commercially available from GE Healthcare Limited, Atto-Tec, Dyomics, Molecular Probes and others. Most such dyes are available as NHS esters.

Methods of conjugating suitable optical reporters (IM), in particular dyes, to amino acids and peptides are described by Licha (*vide supra*), as well as Flanagan *et al* [Bioconj.Chem., 8, 751-756 (1997)]; Lin *et al*, [*ibid*, 13, 605-610 (2002)] and Zaheer [Mol.Imaging, 1(4), 354-364 (2002)]. Methods of conjugating the linker group (L) to the cMBP peptide use analogous chemistry 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 preparation of the pharmaceutical composition of the second aspect, which comprises the imaging agent of the first aspect in sterile, solid form such that, upon reconstitution with a sterile supply of the biocompatible carrier of the second aspect, dissolution occurs to give the desired pharmaceutical composition.

In that instance, the imaging agent, plus other optional excipients as described above, may be provided as a lyophilised powder in a suitable vial or container. The agent is then designed to be reconstituted with the desired biocompatible carrier to the pharmaceutical composition in a sterile, apyrogenic form which is ready for mammalian administration.

A preferred sterile, solid form of the imaging agent is a lyophilised solid. The sterile, solid form is preferably supplied in a pharmaceutical grade container, as described for the pharmaceutical composition (above). When the kit is lyophilised, the formulation may optionally comprise a cryoprotectant chosen from a saccharide, preferably mannitol, maltose or tricine.

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

By the term "optical imaging" is meant any method that forms an image for detection, staging or diagnosis of disease, follow up of disease development or for follow up of disease treatment based on interaction with light in the red to near-infrared region (wavelength 600-1200 nm). Optical imaging further includes all methods from direct visualization without use of any device and involving use of devices such as various scopes, catheters and optical imaging equipment, eg. computer-assisted hardware for tomographic presentations. The modalities and measurement techniques include, but are not limited to: luminescence imaging; endoscopy; fluorescence endoscopy; optical coherence tomography; transmittance imaging; time resolved transmittance imaging; confocal imaging; nonlinear microscopy; photoacoustic imaging; acousto-optical imaging; spectroscopy; reflectance spectroscopy; interferometry; coherence interferometry; diffuse optical tomography and fluorescence mediated diffuse optical tomography (continuous wave, time domain and frequency domain systems), and measurement of light scattering, absorption, polarization, luminescence, fluorescence lifetime, quantum yield, and quenching. Further details of these techniques are provided by: (Tuan Vo-Dinh (editor): "Biomedical Photonics Handbook" (2003), CRC Press LCC; Mycek & Pogue (editors): "Handbook of Biomedical Fluorescence" (2003), Marcel Dekker, Inc.; Splinter & Hopper: "An Introduction to Biomedical Optics" (2007), CRC Press LCC.

The green to near-infrared region light is preferably of wavelength 600-1000 nm. The optical imaging method is preferably fluorescence endoscopy. The mammalian body

of the fifth aspect is preferably the human body. Preferred embodiments of the imaging agent are as described for the first aspect (above). In particular, it is preferred that a fluorescent dye is employed.

In the method of the fifth aspect, the imaging agent or pharmaceutical composition has preferably been previously 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 eg. as an intravenous injection, has already been carried 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 the diagnostic imaging *in vivo* of disease states of the mammalian body where cMet is implicated.

A preferred 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 (CW) excitation. The light excites the reporter molecule (IM). Fluorescence from the imaging agent, which is generated by the excitation light, is detected using a fluorescence detector. The returning 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 processor 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 or chip, such that real-time imaging is possible.

The wavelength for 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 preferred FRI method comprises the steps as follows:

(i) a tissue surface of interest within the mammalian body is illuminated with an excitation light;

(ii) fluorescence from the imaging agent, which is generated by excitation of the imaging moiety (IM), is detected using a fluorescence detector;

(iii) 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 (iii).

In step (i), the excitation light is preferably continuous wave (CW) in nature. In step (iii), the light detected is preferably filtered. An especially preferred FRI method is fluorescence endoscopy.

An alternative imaging method of the fifth aspect uses FDPM (frequency-domain photon migration). This has advantages over continuous-wave (CW) methods where greater depth of detection of the IM within tissue is important [Sevick-Muraca *et al*, Curr.Opin.Chem.Biol., 6, 642-650 (2002)]. For such frequency/time domain imaging, it is advantageous if the IM has fluorescent properties 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-scattering biological tissue of said mammalian body having a heterogeneous composition to light from a light source with a pre-determined 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).

The fluorescence characteristic of step (c) preferably corresponds to uptake of the imaging agent and preferably further comprises mapping a number of quantities corresponding to adsorption and scattering coefficients of the tissue before administration of the imaging agent. The fluorescence characteristic of step (c) preferably corresponds to at least one of fluorescence lifetime, fluorescence quantum efficiency, fluorescence yield and imaging agent uptake. The fluorescence characteristic is preferably independent of the intensity of the emission and independent of imaging agent concentration.

The quantifying of step (c) preferably comprises: (i) establishing an estimate of the values, (ii) determining a calculated emission as a function of the estimate, (iii) comparing the calculated emission to the emission of said detecting to determine an error, (iv) providing a modified estimate of the fluorescence characteristic as a function of the error. The quantifying preferably comprises determining the values from a mathematical relationship modelling multiple light-scattering behaviour of the tissue. The method of the first option preferably further comprises monitoring a metabolic property of the tissue *in vivo* by detecting variation of said fluorescence characteristic.

The optical imaging of the fifth aspect is preferably used to help facilitate the management of colorectal cancer (CRC). By the term "management of CRC" is meant use in the: detection, staging, diagnosis, monitoring of disease progression or the monitoring of treatment. Further details of suitable optical imaging methods have been reviewed by Sevick-Muraca *et al* [Curr.Opin.Chem.Biol., 6, 642-650 (2002)].

In a sixth aspect, the present invention provides a method of detection, staging, diagnosis, monitoring of disease progression or in the monitoring of treatment of colorectal cancer (CRC) of the mammalian body which comprises the *in vivo* optical imaging method of the fifth aspect.

The invention is illustrated by the non-limiting Examples detailed below. Example 1 provides the synthesis of a cMBP peptide of the invention (Compound 1). Example 2

provides the synthesis of a related peptide as a negative control, in which the peptide sequence of Compound 1 is scrambled. Example 3 provides the synthesis of cyanine dye Cy5**, a preferred dye of the invention. Example 4 provides the synthesis of an active ester of Cy5**. Example 5 provides the conjugation of cyanine dyes of the invention to peptides (cMBP peptide and control). Compounds 3 to 7 were compared in this way. Example 6 provides a method of determination of the affinity of the peptides to cMet *in vitro*. The results show that the binding is selective, even when an optical reporter imaging moiety (a cyanine dye) is attached. Example 7 provides data on the *in vivo* testing of Compounds 5 and 7 in an animal model of cancer. Superior tumour : background ratios were seen with Compound 5, whereas Compound 7 (negative control) did not discriminate between tumour and background.

Abbreviations.

Conventional single letter or 3-letter amino acid abbreviations are used.

Acm: Acetamidomethyl

ACN (or MeCN): Acetonitrile

Boc: *tert*-Butyloxycarbonyl

DCM: Dichloromethane

DMF: Dimethylformamide

DMSO: Dimethylsulfoxide

Fmoc: 9-Fluorenylmethoxycarbonyl

HBTU: O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate

HPLC: High performance liquid chromatography

HSPyU O-(N-succinimidyl)-N,N,N′,N′-tetramethyleneuronium hexafluorophosphate

NHS: *N*-hydroxy-succinimide

NMM: *N*-Methylmorpholine

NMP: 1-Methyl-2-pyrrolidinone

Pbf: 2,2,4,6,7-Pentamethyldihydrobenzofuran- 5-sulfonyl

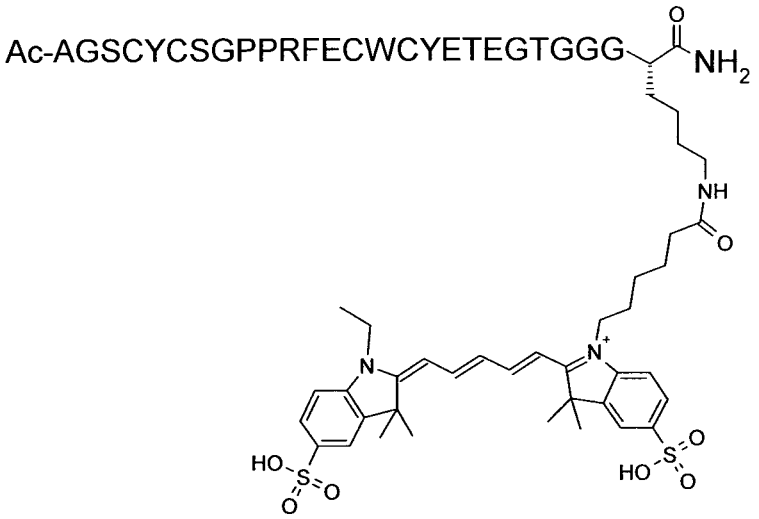
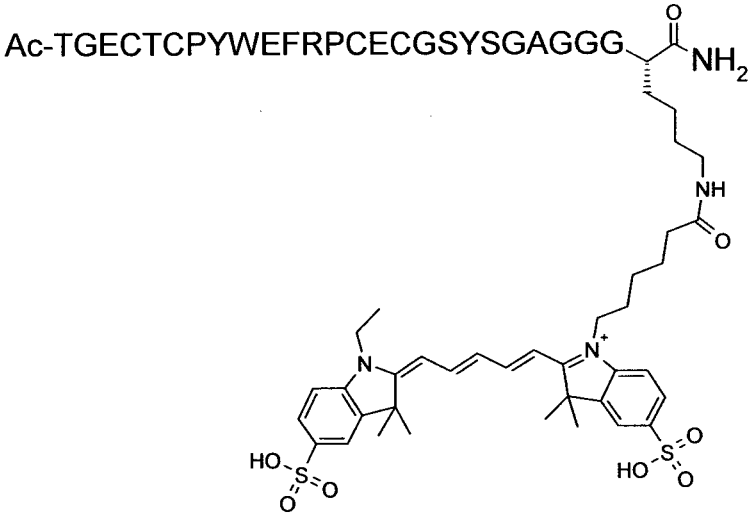
PBS: Phosphate-buffered saline

tBu: t-butyl

TFA: Trifluoroacetic acid

TIS: Triisopropylsilane

Trt: Trityl

No.	Structure of Compounds.
1	Ac-AGSCYCSGPPRFECWCYETEGTGGGK-NH_2
2	Ac-TGECTCPYWEFRPCECGSYSGAGGGK-NH_2 (negative control)
3	 Ac-AGSCYCSGPPRFECWCYETEGTGGGK(ε-Cy5)-NH_2
4	 (negative control)

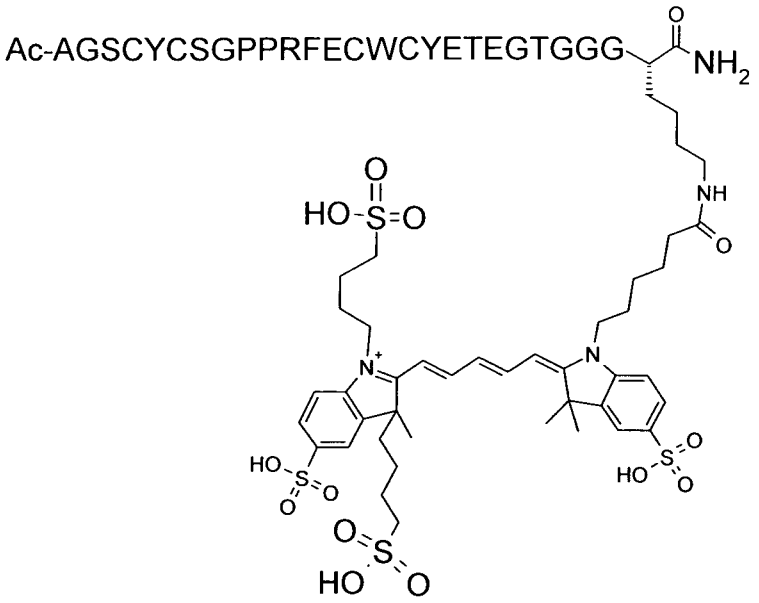
5	 Ac-AGSCYCSGPPRFECWCYETEGTGGGK(ε-Cy5**)-NH_2
6	Ac-AGSCYCSGPPRFECWCYETEGTGGGK(ε-Alexa647)-NH_2
7	Ac-TGECTCPYWEFRPCECGSYSGAGGGK(ε-Cy5**)-NH_2 (negative control).

Table 2: structures of compounds of the invention.

Example 1: Synthesis of Compound 1.

Step (a): synthesis of protected precursor linear peptide.

The precursor linear peptide has the structure:

Ac-Ala-Gly-Ser-Cys-Tyr-Cys(Acm)-Ser-Gly-Pro-Pro-Arg-Phe-Glu-Cys(Acm)-Trp-Cys-Tyr-Glu-Thr-Glu-Gly-Thr-Gly-Gly-Gly-Lys-NH_2

The peptidyl resin H-Ala-Gly-Ser(tBu)-Cys(Trt)-Tyr(tBu)-Cys(Acm)-Ser(tBu)-Gly-Pro-Pro-Arg(Pbf)-Phe-Glu(OtBu)-Cys(Acm)-Trp(Boc)-Cys(Trt)-Tyr(tBu)-Glu(OtBu)-Thr($\psi^{Me,Me}$pro)-Glu(OtBu)-Gly-Thr(tBu)-Gly-Gly-Gly-Lys(Boc)-Polymer 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 pre-activated amino acids (using HBTU) was applied in the coupling steps. Glu-Thr

pseudoproline (Novabiochem 05-20-1122) was incorporated in the sequence. The resin was transferred to a nitrogen bubbler apparatus and treated with a solution of acetic anhydride (1 mmol) and NMM (1 mmol) dissolved in DCM (5 mL) for 60 min. The anhydride solution was removed by filtration and the resin washed with DCM and dried under a stream of nitrogen.

The simultaneous removal of the side-chain protecting groups and cleavage of the peptide from the resin was carried out in TFA (10 mL) containing 2.5 % TIS, 2.5 % 4-thiocresol and 2.5 % water for 2 hours and 30 min. The resin was removed by filtration, TFA removed *in vacuo* and diethyl ether added to the residue. The formed precipitate was washed with diethyl ether and air-dried affording 264 mg of crude peptide.

Purification by preparative HPLC (gradient: 20-30 % B over 40 min where A = H_2O/0.1 % TFA and B = ACN/0.1 % TFA, flow rate: 10 mL/min, column: Phenomenex Luna 5μ C18 (2) 250 x 21.20 mm, detection: UV 214 nm, product retention time: 30 min) of the crude peptide afforded 100 mg of pure Compound 1 linear precursor. The pure product was analysed by analytical HPLC (gradient: 10-40 % B over 10 min where A = H_2O/0.1 % 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: 6.54 min). Further product characterisation was carried out using electrospray mass spectrometry (MH_2^{2+} calculated: 1464.6, MH_2^{2+} found: 1465.1).

Step (b): Formation of Monocyclic Cys4-16 disulfide bridge.

Cys4-16; Ac-Ala-Gly-Ser-Cys-Tyr-Cys(Acm)-Ser-Gly-Pro-Pro-Arg-Phe-Glu-Cys(Acm)-Trp-Cys-Tyr-Glu-Thr-Glu-Gly-Thr-Gly-Gly-Gly-Lys-NH_2.

The linear precursor 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 stirred 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 mL) was injected in portions onto a preparative HPLC column for product purification.

Purification by preparative HPLC (gradient: 0 % B for 10 min, then 0-40 % B over 40

min where A = H_2O/0.1 % TFA and B = ACN/0.1 % TFA, flow rate: 10 mL/min, column: Phenomenex Luna 5μ C18 (2) 250 x 21.20 mm, detection: UV 214 nm, product retention time: 44 min) of the residue afforded 72 mg of pure Compound 1 monocyclic precursor.

The pure product (as a mixture of isomers P1 to P3) was analysed by analytical HPLC (gradient: 10-40 % B over 10 min where A = H_2O/0.1 % 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: 5.37 min (P1); 5.61 min (P2); 6.05 min (P3)). Further product characterisation was carried out using electrospray mass spectrometry (MH_2^{2+} calculated: 1463.6, MH_2^{2+} found: 1464.1 (P1); 1464.4 (P2); 1464.3 (P3)).

Step (c): Formation of Second Cys6-14 disulfide bridge (Compound 1).

The monocyclic precursor from step (b) (72 mg) was dissolved in 75 % AcOH/water (72 mL) under a blanket of nitrogen. 1 M HCl (7.2 mL) and 0.05 M I_2 in AcOH (4.8 mL) were added in that order and the mixture stirred for 45 min. 1 M 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/0.1 % TFA (4 mL) and the product purified using preparative HPLC.

Purification by preparative HPLC (gradient: 0 % B for 10 min, then 20-30 % B over 40 min where A = H_2O/0.1 % TFA and B = ACN/0.1 % TFA, flow rate: 10 mL/min, column: Phenomenex Luna 5μ C18 (2) 250 x 21.20 mm, detection: UV 214 nm, product retention time: 43-53 min) of the residue afforded 52 mg of pure Compound 1. The pure product was analysed by analytical HPLC (gradient: 10-40 % B over 10 min where A = H_2O/0.1 % 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: 6.54 min). Further product characterisation was carried out using electrospray mass spectrometry (MH_2^{2+} calculated: 1391.5, MH_2^{2+} found: 1392.5).

Example 2: Synthesis of Compound 2.

Ac-Thr-Gly-Glu-Cys-Thr-Cys(Acm)-Pro-Tyr-Trp-Glu-Phe-Arg-Pro-Cys(Acm)-Glu-Cys-Gly-Ser-Tyr-Ser-Gly-Ala-Gly-Gly-Gly-Lys-NH_2

Compound 2 is a negative control, where the peptide sequence of Compound 1 has been scrambled.

Step (a): synthesis of protected precursor linear peptide.

The peptidyl resin H-Thr(tBu)-Gly-Glu(OtBu)-Cys(Trt)-Thr(tBu)-Cys(Acm)-Pro-Tyr(tBu)-Trp(Boc)-Glu(OtBu)-Phe-Arg(Pbf)-Pro-Cys(Acm)-Glu(OtBu)-Cys(Trt)-Gly-Ser(tBu)-Tyr(tBu)-Ser($\psi^{Me,Me}$pro)-Gly-Ala-Gly-Gly-Gly-Lys(Boc)-Polymer 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 pre-activated amino acids (using HBTU) was applied in the coupling steps. Tyr-Ser pseudoproline (Novabiochem 05-20-1014) was incorporated in the sequence. The resin was transferred to a nitrogen bubbler apparatus and treated with a solution of acetic anhydride (1 mmol) and NMM (1 mmol) dissolved in DCM (5 mL) for 60 min. The anhydride solution was removed by filtration and the resin washed with DCM and dried under a stream of nitrogen.

The simultaneous removal of the side-chain protecting groups and cleavage of the peptide from the resin was carried out in TFA (10 mL) containing 2.5 % TIS, 2.5 % 4-thiocresol and 2.5 % water for 2 hours and 10 min. The resin was removed by filtration, TFA removed *in vacuo* and diethyl ether added to the residue. The formed precipitate was washed with diethyl ether and air-dried affording 216 mg of crude peptide.

Purification by preparative HPLC (gradient: 20-30 % B over 40 min where A = H_2O/0.1 % TFA and B = ACN/0.1 % TFA, flow rate: 50 mL/min, column: Phenomenex Luna 5μ C18 (2) 250 x 50 mm, detection: UV 214 nm, product retention time: 34.1 min) of the crude peptide afforded pure DX-1662 negative control linear precursor dissolved in 200 mL of ACN/water. The pure product was analysed by analytical HPLC (gradient: 10-40 % B over 5 min where A = H_2O/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, product retention time: 3.52 min). Further product characterisation was carried out using electrospray mass spectrometry (MH_2^{2+}

calculated: 1464.6, MH_2^{2+} found: 1464.9).

Step (b): Formation of Monocyclic Cys4-16 disulfide bridge.

Cys4-16; Ac-Thr-Gly-Glu-Cys-Thr-Cys(Acm)-Pro-Tyr-Trp-Glu-Phe-Arg-Pro-Cys(Acm)-Glu-Cys-Gly-Ser-Tyr-Ser-Gly-Ala-Gly-Gly-Gly-Lys-NH_2

DMSO (10 mL) was added to the negative control linear precursor solution from step (a) (200 mL, see 4.3.1) and the solution adjusted to pH 7 using ammonia. The reaction mixture was heated at 40 °C for 18 hours, then at 60 °C for 60 min. The solution was adjusted to pH 2 using TFA and ACN removed by evaporation *in vacuo*. The residue was subjected to preparative HPLC purification.

Purification by preparative HPLC (gradient: 0 % B for 5 min, then 20-30 % B over 60 min where A = H_2O/0.1 % TFA and B = ACN/0.1 % TFA, flow rate: 50 mL/min, column: Phenomenex Luna 5μ C18 (2) 250 x 50 mm, detection: UV 214 nm, product retention time: 29.6 min) of the residue afforded pure negative control monocyclic precursor in 100 mL of ACN/water. The pure product was analysed by analytical HPLC (gradient: 10-40 % B over 5 min where A = H_2O/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, product retention time: 3.46 min). Further product characterisation was carried out using electrospray mass spectrometry (MH_2^{2+} calculated: 1463.6, MH_2^{2+} found: 1463.7).

Step (c): Formation of Second Cys6-14 disulfide bridge (Compound 2).

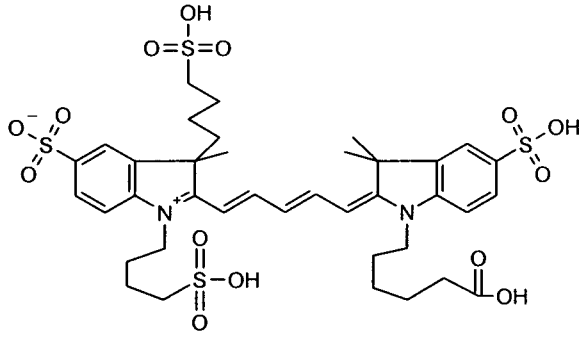
Cys4-16, 6-14; Ac-Thr-Gly-Glu-Cys-Thr-Cys-Pro-Tyr-Trp-Glu-Phe-Arg-Pro-Cys-Glu-Cys-Gly-Ser-Tyr-Ser-Gly-Ala-Gly-Gly-Gly-Lys-NH_2.

The negative control monocyclic precursor solution from step (b) (100 mL) was diluted with AcOH (100 mL). 1 M HCl (5 mL) and 0.05 M I_2 in AcOH (7 mL) were added in that order under a blanket of argon and the mixture stirred for 20 min. 1 M ascorbic acid (1 mL) was added giving a colourless mixture. Most of the solvents were evaporated *in vacuo* and the residue (30 mL) diluted with water/0.1 % TFA (100 mL) and the product purified using preparative HPLC.

Purification by preparative HPLC (gradient: 0 % B for 10 min, then 20-30 % B over

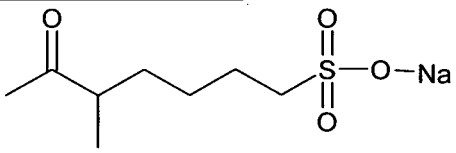
60 min where A = H_2O/0.1 % TFA and B = ACN/0.1 % TFA, flow rate: 50 mL/min, column: Phenomenex Luna 5μ C18 (2) 250 x 50 mm, detection: UV 214 nm, product retention time: 32.8 min) of the residue afforded 30 mg of pure Compound 2. The pure product was analysed by analytical HPLC (gradient: 10-40 % B over 10 min where A = H_2O/0.1 % 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: 6.54 min). Further product characterisation was carried out using electrospray mass spectrometry (MH_2^{2+} calculated: 1391.5, MH_2^{2+} found: 1392.5).

Example 3: Synthesis of the Cyanine Dye 2-{(1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-3,3-dimethyl-5-sulfo-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl}-3-methyl-1,3-*bis*(4-sulfobutyl)-3*H*-indolium-5-sulfonate (Cy5).**



Cy5**

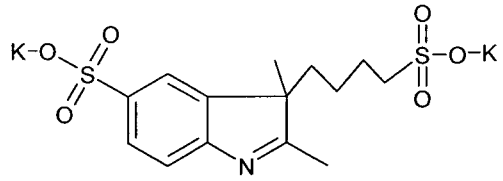
(3a) 5-Methyl-6-oxoheptane-1-sulfonic acid.



Ethyl 2-methylacetoacetate (50g) in DMF (25ml) was added to a suspension of sodium hydride (12.0g of 60% NaH in mineral oil) in DMF (100ml), dropwise with ice-bath cooling over 1 hour, (internal temperature 0-4°C). This mixture was allowed to warm to ambient temperature for 45mins with stirring before re-cooling. A solution of 1,4-butanesultone (45g) in DMF (25ml) was then added dropwise over 15 minutes. The final mixture was heated at 60°C for 18hours. The solvent was removed by rotary evaporation and the residue partitioned between water and diethyl ether. The aqueous layer was collected, washed with fresh diethyl ether and rotary evaporated to yield a sticky foam. This intermediate was dissolved in water (100ml) and sodium hydroxide (17.8g) added over 15 minutes with stirring. The mixture was heated at 90°C for 18 hours. The cooled reaction mixture was adjusted to ~pH2 by the addition of concentrated hydrochloric acid (~40ml). The solution was rotary evaporated and dried

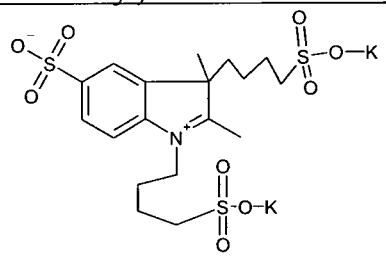
under vacuum. The yellow solid was washed with ethanol containing 2% hydrochloric acid (3x150ml). The ethanolic solution was filtered, rotary evaporated and dried under vacuum to yield a yellow solid. Yield 70g.

(3b) 2,3-Dimethyl-3-(4-sulfobutyl)-3*H*-indole-5-sulfonic acid, dipotassium salt.



4-Hydrazinobenzenesulfonic acid (40g), 5-methyl-6-oxoheptane-1-sulfonic acid (from 3a; 60g) and acetic acid (500ml) were mixed and heated under reflux for 6hrs. The solvent was filtered, rotary evaporated and dried under vacuum. The solid was dissolved in methanol (1L). To this was added 2M methanolic potassium hydroxide (300ml). The mixture was stirred for 3 hours and then the volume of solvent reduced by 50% using rotary evaporation. The resulting precipitate was filtered, washed with methanol and dried under vacuum. Yield 60g. MS (LCMS) : MH^+ 362. Acc. Mass: Found, 362.0729. $MH^+ = C_{14}H_{20}NO_6S_2$ requires m/z 362.0732 (-0.8ppm).

(3c) 2,3-Dimethyl-1,3-*bis*(4-sulfobutyl)-3H-indolium-5-sulfonate, dipotassium salt.



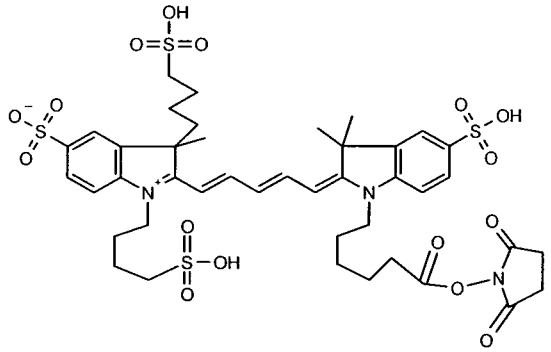
2,3-Dimethyl-3-(4-sulfobutyl)-3*H*-indole-5-sulfonic acid (from 3b; 60g) was heated with 1,4 butane sultone (180g) and tetramethylene sulfone (146ml) at 140°C for 16 hours. The resulting red solid was washed with diethyl ether, ground into a powder and dried under vacuum. Yield 60g

(3d) Cy5**, as TFA salt.

1-(5'-Carboxypentyl)-2,3,3-trimethyl-indolenium bromide-5-sulfonic acid, K^+ salt (2.7g), malonaldehyde *bis*(phenylimine) monohydrochloride (960mg), acetic anhydride (36ml) and acetic acid (18ml) were heated at 120°C for 1 hour to give a dark brown-red solution. The reaction mixture was cooled to ambient temperature. 2,3-Dimethyl-1,3-*bis*(4-sulfobutyl)-3H-indolium-5-sulfonate (from 3c; 8.1g) and potassium acetate (4.5g) were added to the mixture, which was stirred for 18 hours at ambient temperature. The resulting blue solution was precipitated using ethyl acetate and dried under vacuum. The crude dye was purified by liquid chromatography

(RPC$_{18}$. Water + 0.1% TFA/ MeCN + 0.1%TFA gradient). Fractions containing the principal dye peak were collected, pooled and evaporated under vacuum to give the title dye, 2g. UV/Vis (Water+0.1%TFA): 650nm. MS (MALDI-TOF): MH+ 887.1. $MH^+ = C_{38}H_{50}N_2O_{14}S_4$ requires m/z 887.1.

Example 4: Synthesis of 2-[(1*E*,3*E*,5*E*)-5-(1-{6-[(2,5-dioxopyrrolidin-1-yl)oxy]-6-oxohexyl}-3,3-dimethyl-5-sulfo-1,3-dihydro-2*H*-indol-2-ylidene)penta-1,3-dienyl]-3-methyl-1,3-bis(4-sulfobutyl)-3*H*-indolium-5-sulfonate, diisopropylethylamine salt (NHS Ester of Cy5).**



Cy5** (Example 3; 10mg) was dissolved in anhydrous DMSO (3ml); to this were added HSPyU (20mg) and N,N′-diisopropylethylamine (80µl). The resulting solution was mixed for 3 hours, whereupon TLC (RPC18. Water/MeCN) revealed complete reaction. The dye was isolated by precipitation in ethyl acetate/diethyl ether, filtered, washed with ethyl acetate and dried under vacuum. UV/Vis (Water) 650nm. MS (MALDI-TOF) MH+ 983.5. $MH^+ = C_{42}H_{53}N_3O_{16}S_4$ requires m/z 984.16.

Example 5: Conjugation of Dyes, Synthesis of Compounds 3 to 7.

Cys4-16, 6-14; Ac-Ala-Gly-Ser-Cys-Tyr-Cys-Ser-Gly-Pro-Pro-Arg-Phe-Glu-Cys-Trp-Cys-Tyr-Glu-Thr-Glu-Gly-Thr-Gly-Gly-Gly-Lys(Cy5)-NH_2 (Compound 3).

Compound 1 (10 mg), NMM (4 µL) and Cy5 NHS ester (5.7 mg; GE Healthcare PA15104) were dissolved in NMP (1 mL) and the reaction mixture stirred for 7 hrs. The reaction mixture was then diluted with 5 % ACN/water (8 mL) and the product purified using preparative HPLC.

Purification by preparative HPLC (gradient: 5-50 % B over 40 min where A = H_2O/0.1 % HCOOH and B = ACN/0.1 % HCOOH, flow rate: 10 mL/min, column: Phenomenex Luna 5µ C18 (2) 250 x 21.20 mm, detection: UV 214 nm, product

retention time: 35.5 min) of the crude peptide afforded 8.1 mg of pure Compound 3. The pure product was analysed by analytical HPLC (gradient: 5-50 % B over 10 min where A = H_2O/0.1 % HCOOH and B = ACN/0.1 % HCOOH, flow rate: 0.3 mL/min, column: Phenomenex Luna 3µ C18 (2) 50 x 2 mm, detection: UV 214 nm, product retention time: 8.15 min). Further product characterisation was carried out using electrospray mass spectrometry (MH_2^{2+} calculated: 1710.6, MH_2^{2+} found: 1711.0).

Compound 4 was prepared in a similar manner - electrospray mass spectrometry (MH_2^{2+} calculated: 1710.6, MH_2^{2+} found: 1710.9).

Other dye-peptide conjugates (Compounds 5 to 7) were prepared by analogous methods. Alexa647was purchased from Molecular Probes (A20106):
Compound 5 (MH_2^{2+} calculated: 1825.7, MH_2^{2+} found: 1825.9),
Compound 6 (MH_2^{2+} calculated: 1811.7, MH_2^{2+} found: 1812.0),
Compound 7 (MH_2^{2+} calculated: 1825.7, MH_2^{2+} found: 1826.2).

Example 6: *In Vitro* Fluorescence polarisation assay.

The principle of the fluorescence polarisation method can briefly be described as follows:

Monochromatic light passes through a horizontal polarizing filter and excites fluorescent molecules in the sample. Only those molecules that oriented properly 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 anistropy value (A), is the ratio between the light intensities following the equation

$$A = \frac{\textit{Intensity with horizontal polarizer - Intensity with vertical polarizer}}{\textit{Intensity with horizontal polarizer + 2* Intensity with vertical polarizer}}$$

The fluorescence anistropy measurements were performed in 384-well microplates in a volume of 10 µL in binding buffer (PBS, 0.01%Tween-20, pH 7.5) using a Tecan Safire fluorescence polarisation plate reader (Tecan , US) at ex646/em678 nm. The concentration of dye-labelled peptide was held constant (20nM) and the concentrations of the human or mouse c-Met/ Fc chimera (R&D Systems) or Semaphorin 6A (R&D Systems) were varied from 0-150 nM. Binding mixtures were equilibrated in the microplate for 10 min at 30°C. The observed change in anistropy was fit to the equation

$$r_{obs} = r_{free} + (r_{bound} - r_{free}) \frac{(K_D + cMet + P) - \sqrt{(K_D + cMet + P)2 - 4 \cdot cMet \cdot P}}{2 \cdot P}$$

where *robs* is the observed anistropy, *rfree* is the anistropy of the free peptide, *rbound* is the anistropy of the bound peptide, K_D is the dissociation constant, cMet 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 1:1 stoichiometry. Data fitting was done *via* nonlinear regression using GraphPad Prism software to obtain the K_D value (one-site binding).

Compounds 3 and 4 were tested for binding towards human and mouse c-Met (Fc chimera). The results showed a K_D of 3 +/- 1 nM for the binding of Compound 3 to human c-Met. There was no binding of Compound 4 to human c-Met. Furthermore, Compounds 3 and 4 showed no binding to mouse c-Met in the tested range.

Using the same method, Compound 5 was found to have a K_D for human cMet of 1.1 nM.

Example 7: *In Vivo* testing of Compounds 5 and 7.

(a) Animal Model.

54 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 is an inbred immunocompromised mouse strain with a high take rate for human tumours as compared to other nude mice strains. The mice were 4 weeks old upon arrival and with a body weight of approx. 20 grams at the start of the study. The animals were housed in individually ventilated cages (IVC, Scanbur BK) with HEPA filtered air. The animals had ad libitum access to "Rat and Mouse nr. 3 Breeding" diet (Scanbur BK) and tap water acidified by addition of HCl to a molar concentration of 1 mM (pH 3.0).

The colon cancer cell HCT-15 is derived from human colon carcinomas and is reported to express c-Met according to Zeng *et al* [Clin. Exp. Metastasis, 21, 409-417. (2004)]. The cell line was proven to be tumorigenic when inoculated subcutaneously into nude mice [Flatmark *et al*, Eur. J. Cancer 40,1593-1598 (2004)].

HCT-15 cells were grown and prepared for subcutaneous inoculation in RPMI (Sigma

Cat # R0883) with 10% serum and penicillin/streptomycin. Stocks were made at passage number four (P4) and frozen down for storage in liquid nitrogen at $3x10^7$ cells/vial in the culture media containing 5% DMSO. On the day of the transplantation, the cells were thawed quickly in a 37°C water bath (approx. 2 min), washed and resuspended in PBS/2% serum (centrifugation at 1200 rpm for 10 min). Thorough mixing of cells in the vials was ensured every time cells were aspirated into the dosing syringe. Volumes of 0.1 ml of cell suspension were injected s.c. at the shoulder and at the back using a fine bore needle (25 G) while the animals were under light gas anaesthesia. The animals were then returned to their 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 printer paper was imaged to obtain a flat field image which was used to correct for illumination inhomogeneities. The test substances were injected intravenously in the lateral tail vein during physical fixation. The injection volume was 0.1ml, which corresponds to a dose of 1nmol test substance per animal. After injection the animals were returned to their cages. The animals were sacrificed immediately before imaging by cervical dislocation. The optimal imaging time point for each test substance was estimated based on comparison of wash out rates in the skin and in muscle tissue in a limited number of animals (n=1-6). The imaging time point for Compounds 3 and 4 was 120 minutes post injection. For each animal the subcutaneously grown tumours were excised post mortem. A thin slice, approximately 1.6mm thick and 3-4mm in diameter, was cut off the edge of one of the tumours. The tumour slice was then imaged against an area of normal colon from the same animal.

(c) Imaging.

Imaging was performed through a clinical laparoscope adapted to use a light source to excite the reporter and a filtering system to extract the fluorescence component. A 635nm laser was used for excitation of the reporter molecule. A Hamamatsu ORCA ERG CCD camera was used as the detector. The camera was operated in 2x2 binning mode with 0 gain. Standard exposure time for colon imaging was 10s. System calibration measurements indicate that the 10s exposure time with the animal imaging

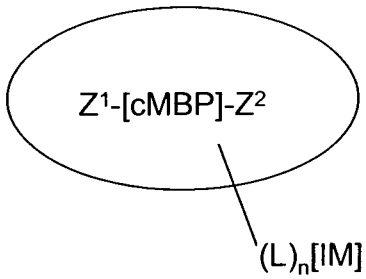
system corresponds to 40ms exposure with a clinically relevant light source, field of view, and distance to the tissue surface. The intensity distribution in the image was corrected for illumination inhomogeneities through system calibration data. A target to background ratio was computed from regions of interest placed over the tumour, and normal colon background. The images were visually scored using the standard scoring system employed for receiver operating characteristic analysis.

(d) Results.

Compound 5 had a tumour to normal ratio of 1.46:1 and the corresponding scrambled control peptide with the same dye (Compound 7) had a ratio of 1.04:1. Compound 5 had a readily identifiable tumour, whereas nothing was discernible against background with Compound 7.

CLAIMS.

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



(I)

where:

Z^1 is attached to the N-terminus of cMBP, and is H or M^{IG};

Z^2 is attached to the C-terminus of cMBP and is OH, OB^c, or M^{IG},

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-1):

Cys^a-X^1-Cys^c-X^2-Gly-Pro-Pro-X^3-Phe-Glu-Cys^d-Trp-Cys^b-Tyr-X^4-X^5-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^{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^{IG} is a metabolism inhibiting group which is a biocompatible group which inhibits or suppresses *in vivo* metabolism of the peptide;

L is a synthetic linker group of formula $-(A)_m-$ wherein each A is independently $-CR_2-$, $-CR=CR-$, $-C\equiv C-$, $-CR_2CO_2-$, $-CO_2CR_2-$, -NRCO-, -CONR-, -NR(C=O)NR-, -NR(C=S)NR-, $-SO_2NR-$, $-NRSO_2-$, $-CR_2OCR_2-$, $-CR_2SCR_2-$, $-CR_2NRCR_2-$, a C_{4-8} cycloheteroalkylene group, 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 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;

IM is an optical reporter imaging moiety suitable for imaging the mammalian body *in vivo* using light of green to near-infrared wavelength 600-1200 nm.

2. The imaging agent of either of Claim 1, where in addition to SEQ-1, the cMBP further comprises an Asp or Glu residue within 4 amino acid residues of either the C- or N- cMBP peptide terminus, and $-(L)_nIM$ 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, where 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 $-(L)_nIM$ 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:

Ser-Cys^a-X^1-Cys^c-X^2-Gly-Pro-Pro-X^3-Phe-Glu-Cys^d-Trp-Cys^b-Tyr-X^4-X^5-X^6. (SEQ-2);

Ala-Gly-Ser-Cys^a-X^1-Cys^c-X^2-Gly-Pro-Pro-X^3-Phe-Glu-Cys^d-Trp-Cys^b-Tyr-X^4-X^5-X^6-Gly-Thr (SEQ-3).

5. The imaging agent of any one of Claims 1 to 4, wherein X^3 is Arg.

6. The imaging agent of any one of Claims 1 to 5, wherein in addition to SEQ-1, 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):

Ala-Gly-Ser-Cys^{a}-Tyr-Cys^{c}-Ser-Gly-Pro-Pro-Arg-Phe-Glu-Cys^{d}-Trp-Cys^{b}-Tyr-Glu-Thr-Glu-Gly-Thr-Gly-Gly-Gly-Lys.

8. The imaging agent of any one of Claims 1 to 7, where both Z^1 and Z^2 are independently M^{IG}.

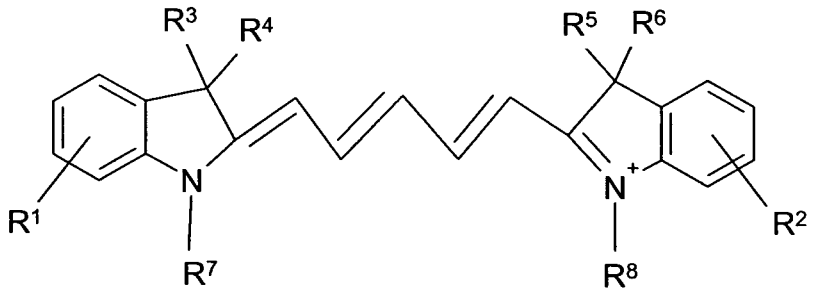
9. The imaging agent of Claim 8, where Z^1 is acetyl and Z^2 is a primary amide.

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

11. The imaging agent of any one of Claims 1 to 10, where IM is a dye having an absorbance maximum in the range 600-1000 nm.

12. The imaging agent of any one of Claim 11, where IM is a cyanine dye.

13. The imaging agent of Claim 12, where the cyanine dye is of Formula III:



(III)

where:

R^1 and R^2 are independently H or SO_3M^1, and at least one of R^1 and R^2 is SO_3M^1, where M^1 is H or B^c;

R^3 and R^4 are independently C_{1-4} alkyl or C_{1-6} carboxyalkyl;

R^5, R^6, R^7 and R^8 are independently R^a groups;

wherein R^a is C_{1-4} alkyl, C_{1-6} carboxyalkyl or $-(CH_2)_kSO_3M^1$, where k

is an integer of value 3 or 4;

with the proviso that the cyanine dye has a total of 1 to 4 SO_3M^1 substituents in the R^1, R^2 and R^a groups.

14. The imaging agent of any one of Claims 1 to 13, where cMBP is as defined in Claim 7, Z^1 and Z^2 are as defined in Claim 9 and IM is as defined in Claims 3 and 13.

15. A pharmaceutical composition which comprises the imaging agent of any one of Claims 1 to 14 together with a biocompatible carrier, in a form suitable for mammalian administration.

16. The pharmaceutical composition of Claim 15, which has a dosage suitable for a single patient and is provided in a suitable syringe or container.

17. A method of preparation of the imaging agent of Claims 1 to 14, which comprises one of steps (i) to (iv):

(i) reaction of a cMBP peptide of formula Z^1-[cMBP]-Z^2 wherein Z^1 is H and Z^2 is a M^{IG} with a compound of formula Y^1-$(L)_n$-[IM], to give the imaging agent of Formula I wherein [IM] is conjugated at the Z^1 position;

(ii) reaction of a cMBP peptide of formula Z^1-[cMBP]-Z^2 wherein $Z^1 = Z^2 = M^{IG}$ and cMBP comprises an Asp or Glu residue within 4 amino acid residues of either the C- or N- cMBP peptide terminus, and all other Asp/Glu residues of the cMBP peptide are protected, with a compound of formula Y^2-$(L)_n$-[IM], to give the imaging agent of Formula I wherein [IM] is conjugated at said Asp or Glu residue of the cMBP peptide;

(iii) reaction of a cMBP peptide of formula Z^1-[cMBP]-Z^3 wherein Z^1 is M^{IG} and Z^3 is a Z^2 group or an activated ester and all other Asp/Glu residues of the cMBP peptides are protected, with a compound of formula Y^2-$(L)_n$-[IM], to give the imaging agent of Formula I wherein [IM] is conjugated at the Z^2 position;

(iv) reaction of a cMBP peptide of formula Z^1-[cMBP]-Z^2 wherein $Z^1 = Z^2 = M^{IG}$ and cMBP comprises a Lys within 4 amino acid residues of either the C- or N- cMBP peptide terminus, with a compound of formula Y^1-$(L)_n$-[IM], to give the imaging agent of Formula I wherein [IM] is conjugated at a Lys

residue of the cMBP peptide;

wherein Z^1, cMBP, Z^2, M^{IG}, L, n and IM are as defined in Claim 1, and

Z^3 is a Z^2 group or an activated ester;

Y^1 is a carboxylic acid, activated ester, isothiocyanate or thiocyanate group;

Y^2 is an amine group.

18. The method of Claim 17, where the reaction of step (iv) is used.

19. A kit for the preparation of the pharmaceutical composition of Claim 15 or 16, which comprises the imaging agent of Claims 1 to 14 in sterile, solid form such that upon reconstitution with a sterile supply of the biocompatible carrier of Claim 15 or 16, dissolution occurs to give the desired pharmaceutical composition.

20. The kit of Claim 19, where the sterile, solid form is a lyophilised solid.

21. A method of *in vivo* optical imaging of the mammalian body which comprises use of either the imaging agent of Claims 1 to 14 or the pharmaceutical composition of Claims 15 or 16 to obtain images of sites of cMet over-expression or localisation *in vivo*.

22. The method of Claim 21, where the imaging agent of Claims 1 to 14 or the pharmaceutical composition of Claims 15 or 16 has been previously administered to said mammalian body.

23. The method of Claim 22, which comprises the steps of:

(i) a tissue surface of interest within the mammalian body is illuminated with an excitation light;

(ii) fluorescence from the imaging agent, which is generated by excitation of the imaging moiety (IM), is detected using a fluorescence detector;

(iii) 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 (iii).

24. The method of Claim 23 where the excitation light of step (i) is continuous wave (CW) in nature.

25. The method of Claim 22, which comprises:

(a) exposing light-scattering biologic tissue of said mammalian body having a heterogeneous composition to light from a light source with a pre-determined 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).

26. The method of any one of Claims 21 to 25, where the optical imaging method comprises fluorescence endoscopy.

27. The method of any one of Claims 21 to 26, 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).

28. 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 21 to 26.