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WO 2014/064258 A **WO 2014/053622 A**
WO 2013/150338 A **WO 2011/131693 A**
WO 2010/123369 A **WO 2009/054725 A**
WO 1989/003849 A
JACS, Vol 136, 2014, GJ Hilinski et al, "Stitched a-helical peptides via bis ring-closing metathesis", 12314- 12322
Biopolymers, Vol 73, 2004, IA Kozlov et al, "Efficient strategies for the conjugation of oligonucleotides to antibodies enabling highly sensitive protein detection", 621-630

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Other: **None**

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GB 2545898 B

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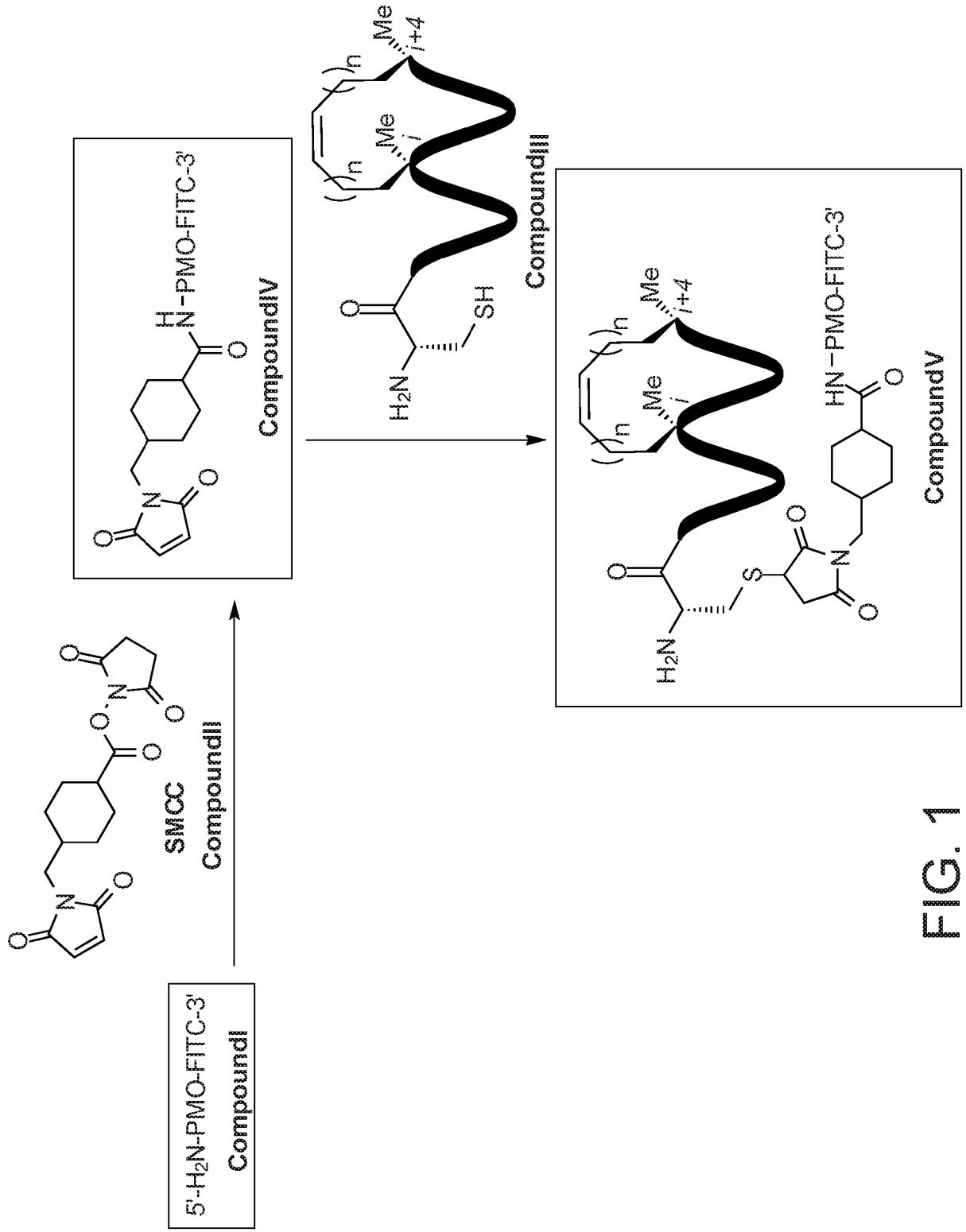
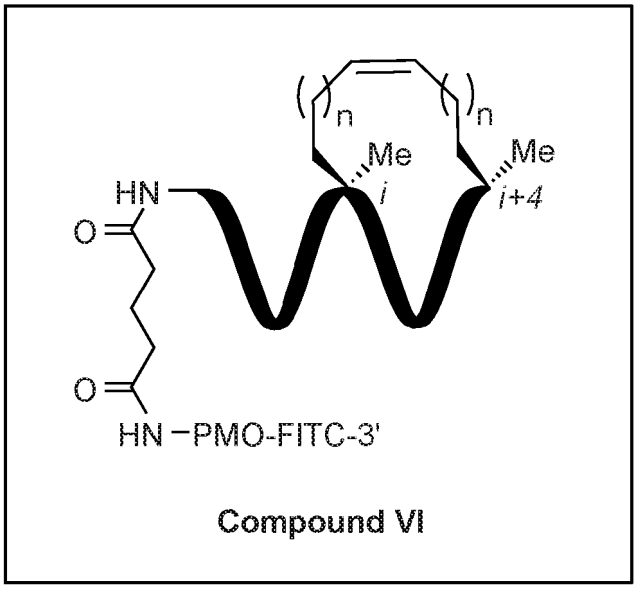
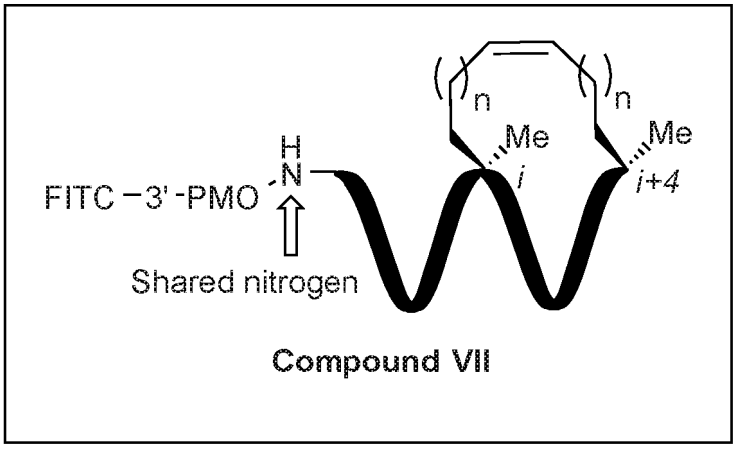

5'-H2N-PMO-FITC-3'
CompoundI
SMCC
CompoundII
H
N-PMO-FITC-3'
O
CompoundIV
Me
i+4
n
Me
i
n
O
SH
H2N
CompoundIII
Me
i+4
n
Me
i
n
H2N
O
S
O
N
O
HN-PMO-FITC-3'
O
CompoundV

FIG. 1



Compound VI

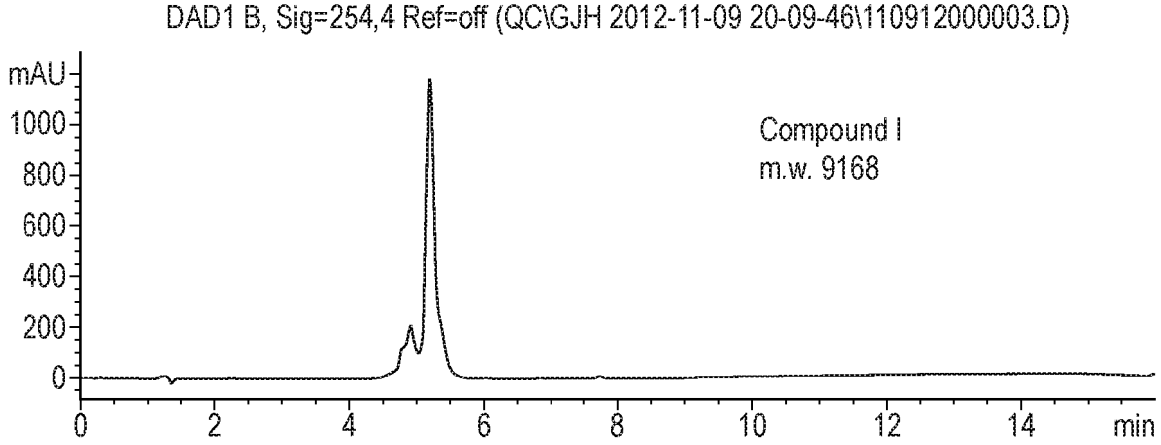
FIG. 2

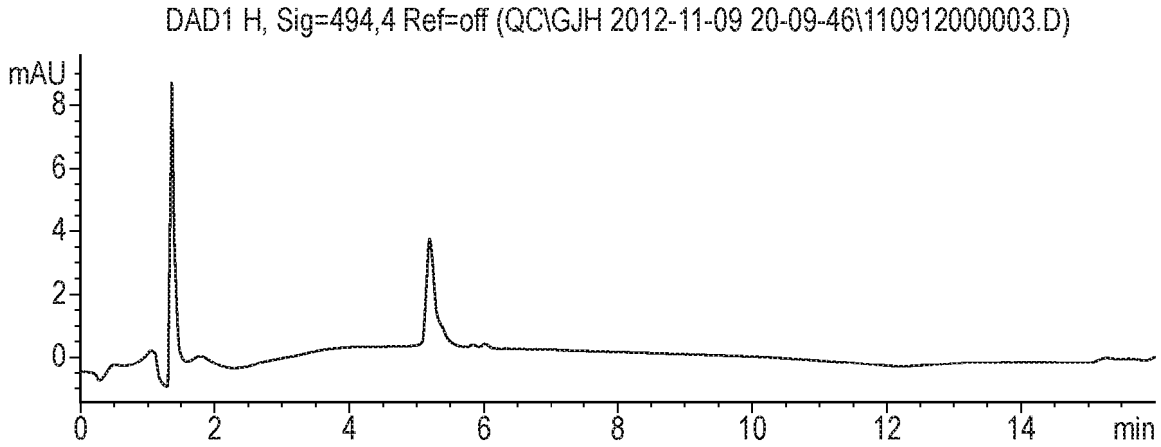


Compound VII

FIG. 3

a) FITC Labelled PMO





MS Spectrum

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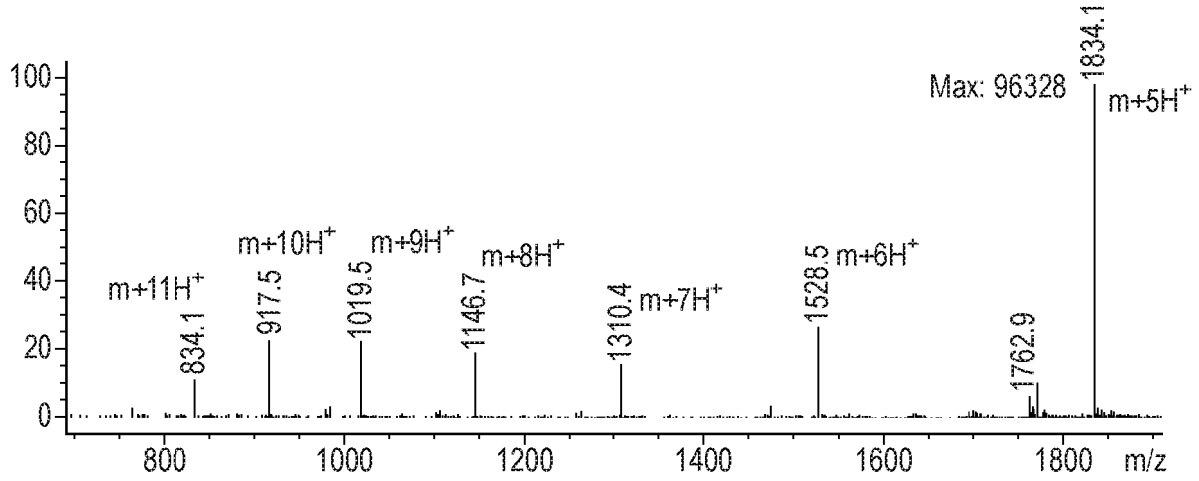
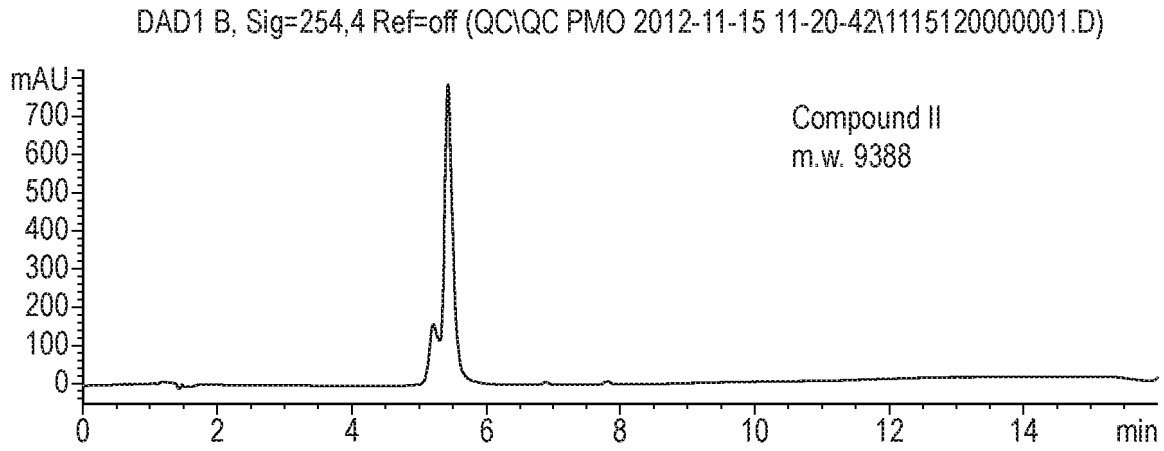
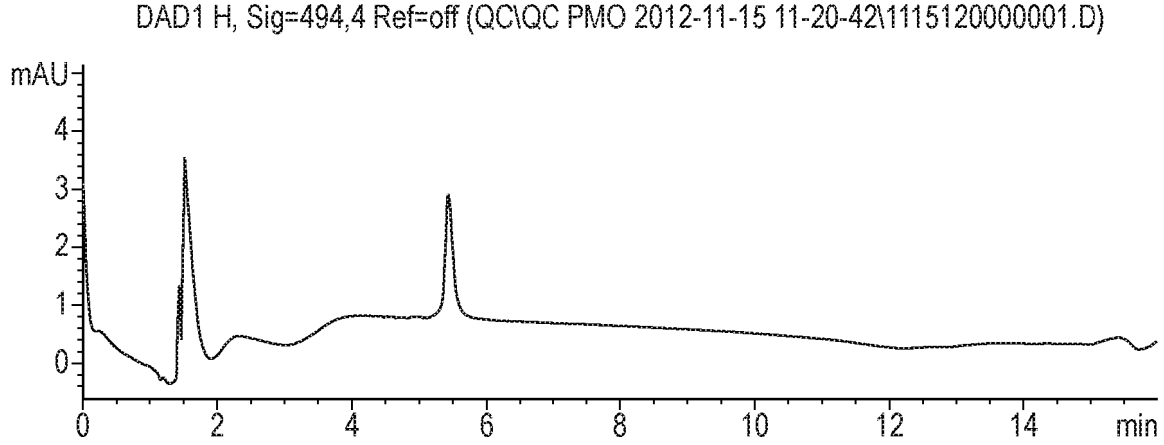


FIG. 4

b) SMCC Linked PMO





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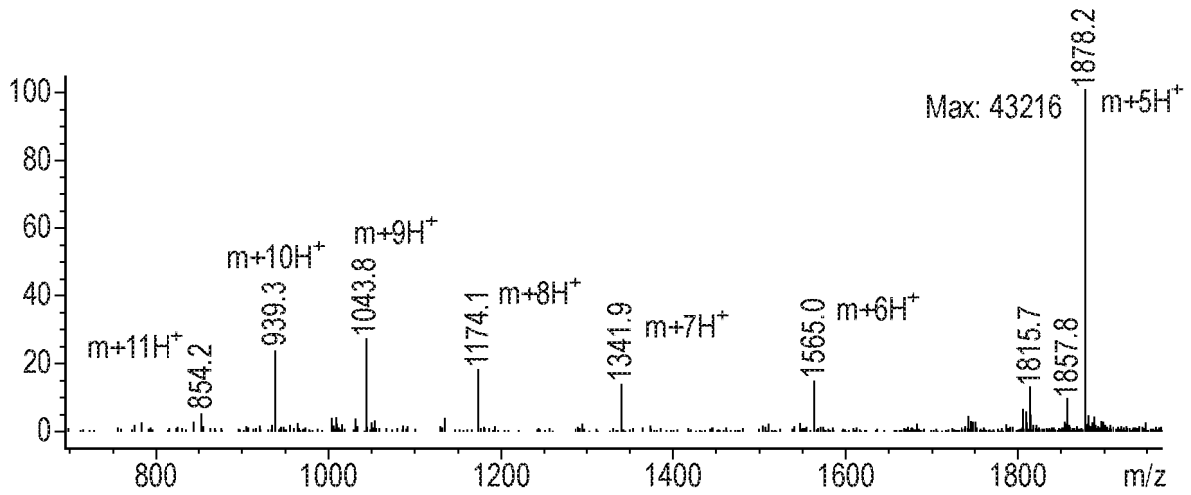
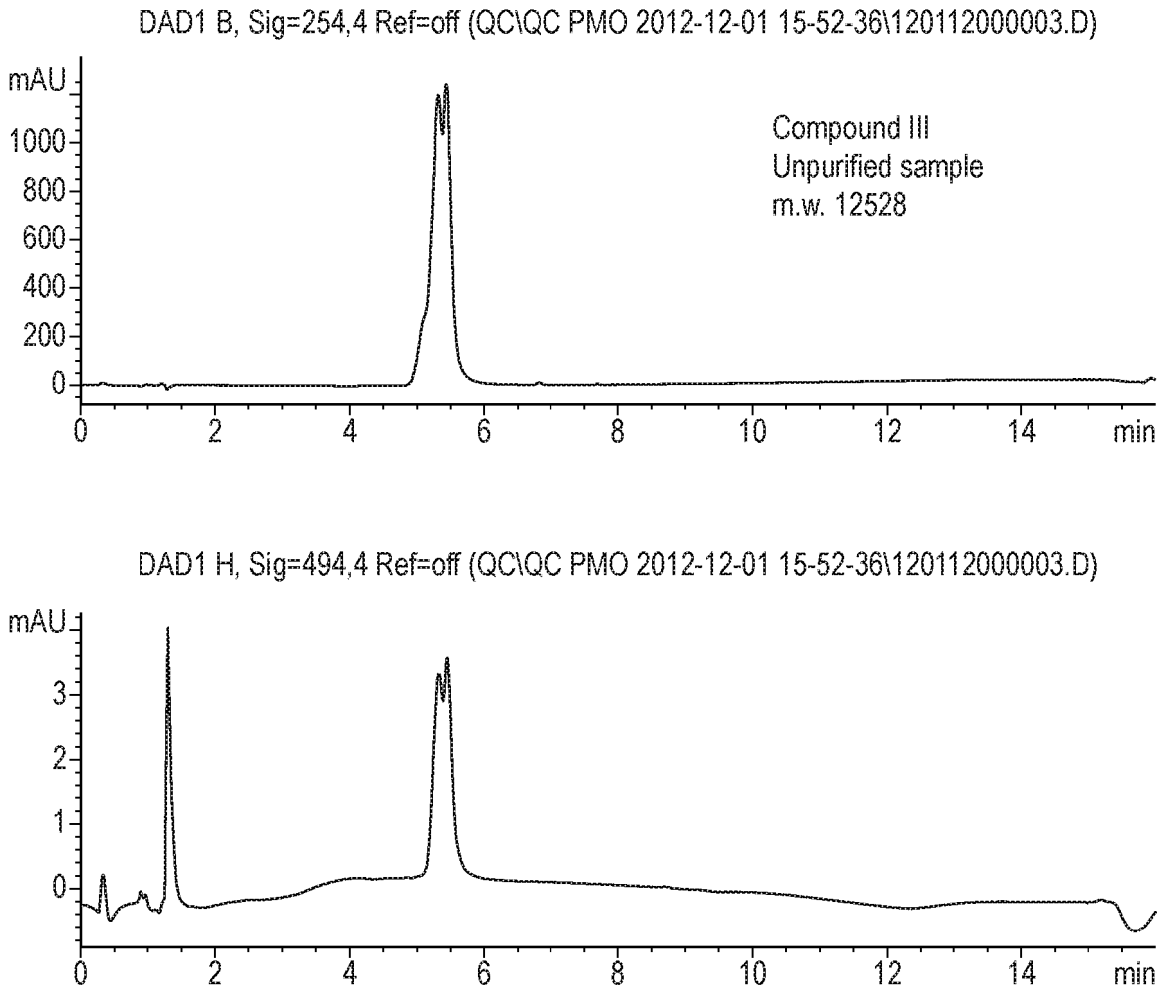


FIG. 4 Cont'd

c) PMO-SMCC-StaP



MS Spectrum

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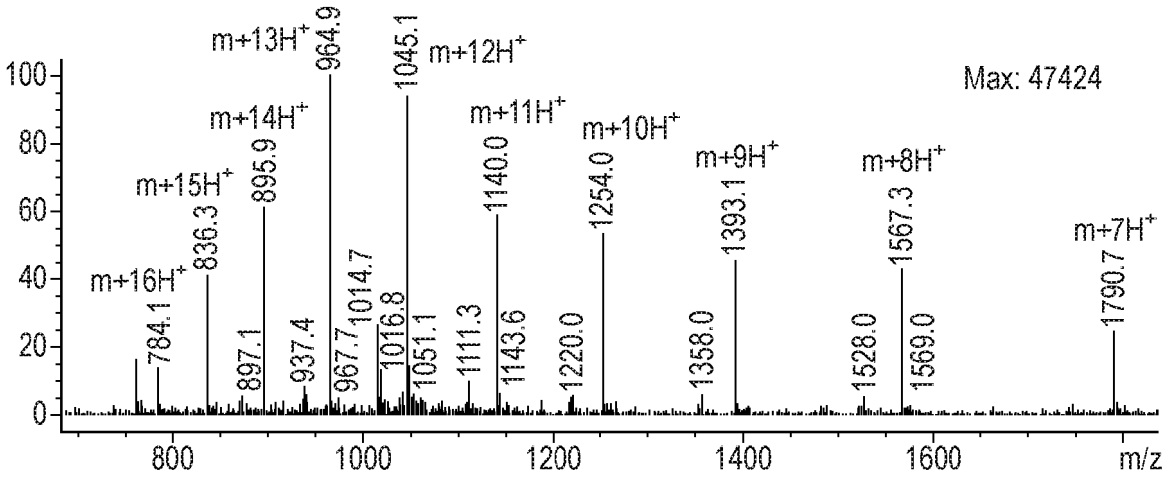


FIG. 4 Cont'd

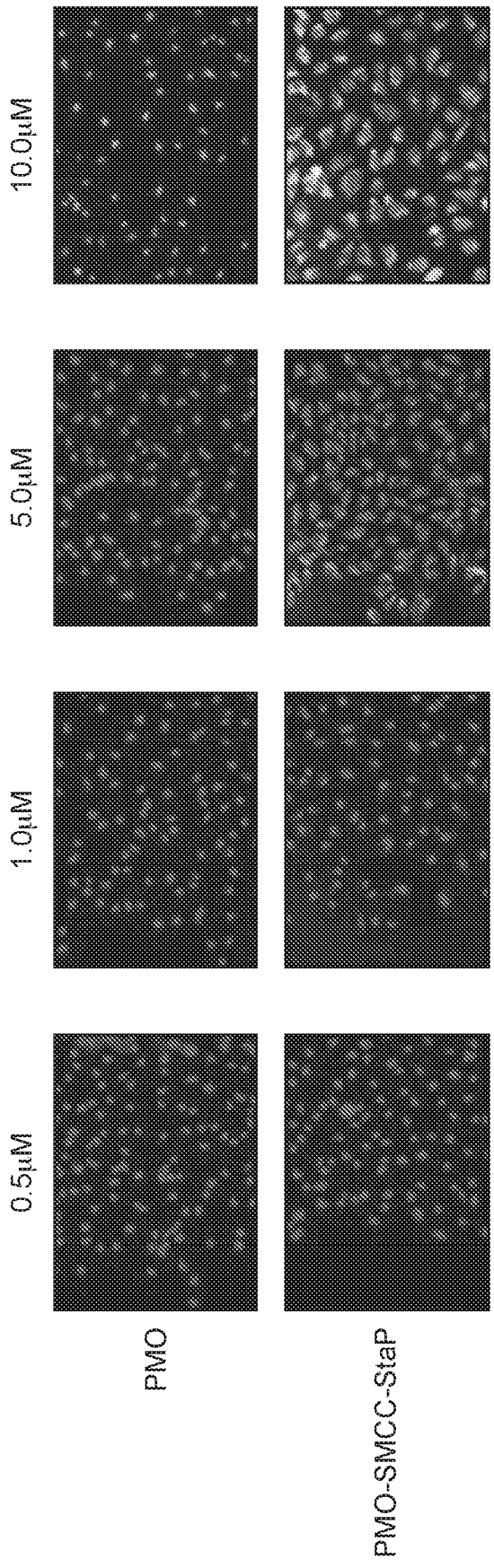


FIG. 5

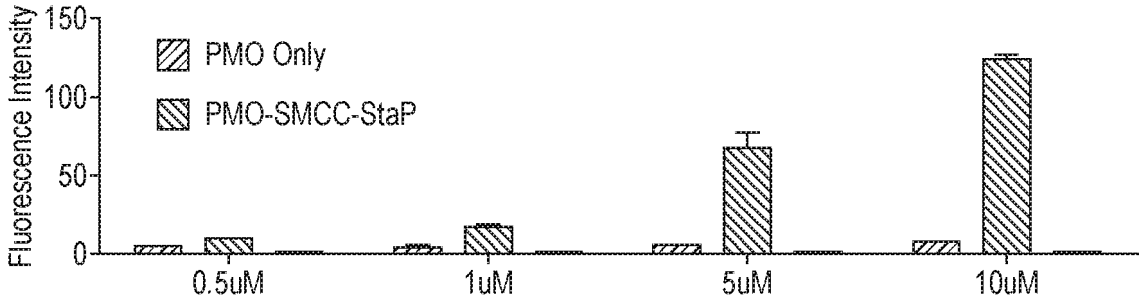

Fluorescence Intensity
150
100
50
0
PMO Only
PMO-SMCC-StaP
0.5uM
1uM
5uM
10uM

FIG. 6

21 03 17

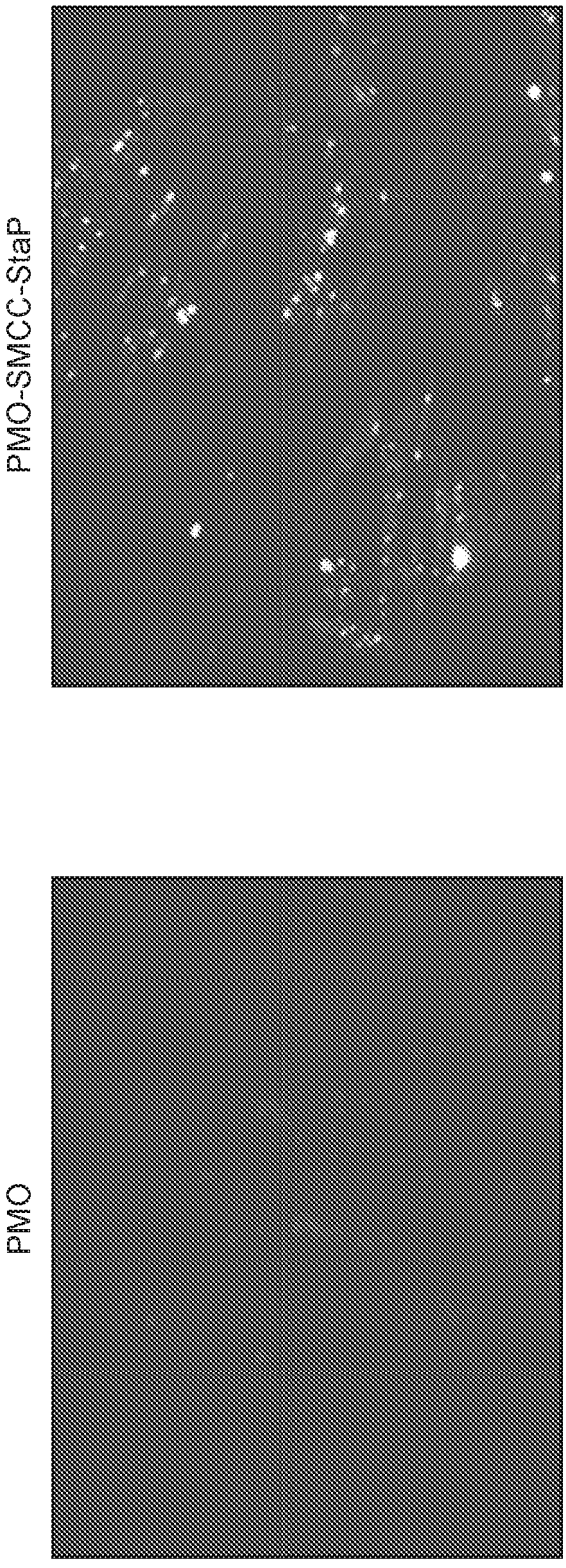


FIG. 7

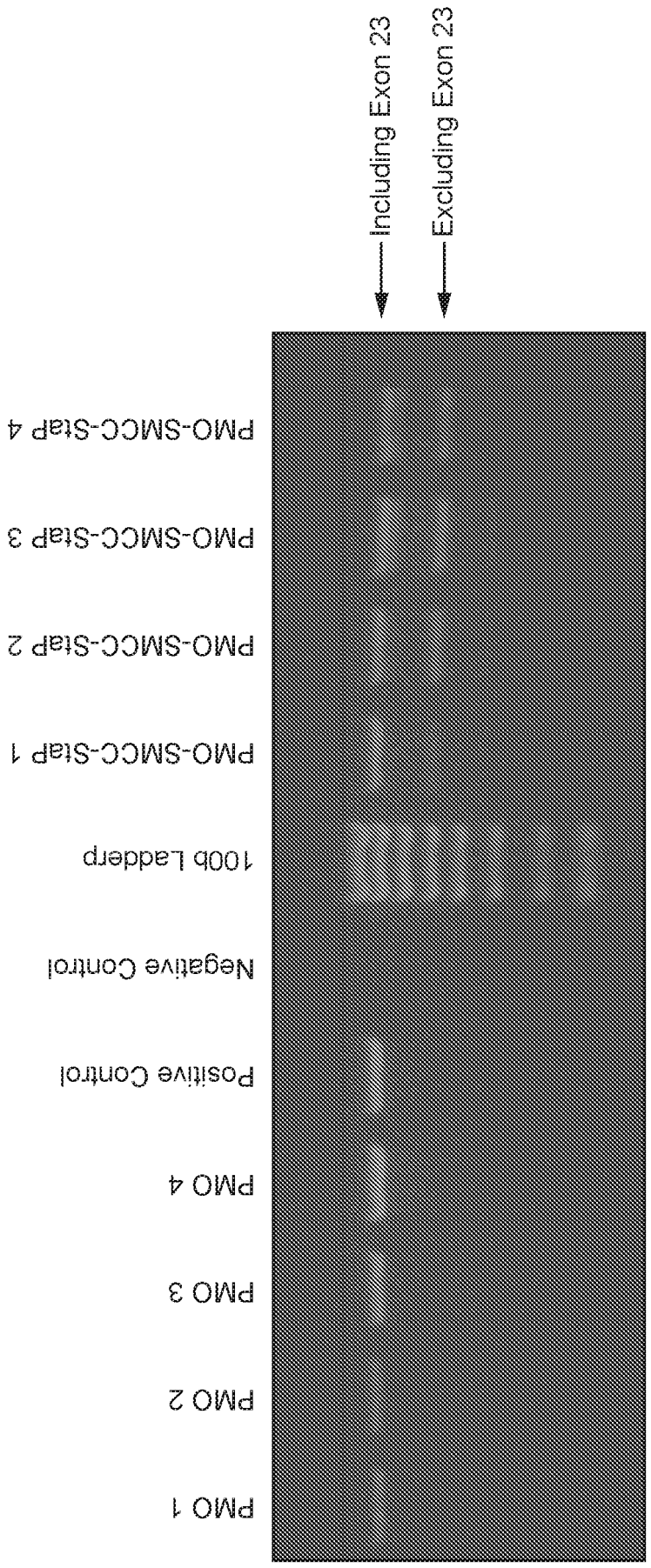

Including Exon 23
Excluding Exon 23
PMO-SMCC-StaP 4
PMO-SMCC-StaP 3
PMO-SMCC-StaP 2
PMO-SMCC-StaP 1
100b Ladderp
Negative Control
Positive Control
PMO 4
PMO 3
PMO 2
PMO 1

FIG. 8

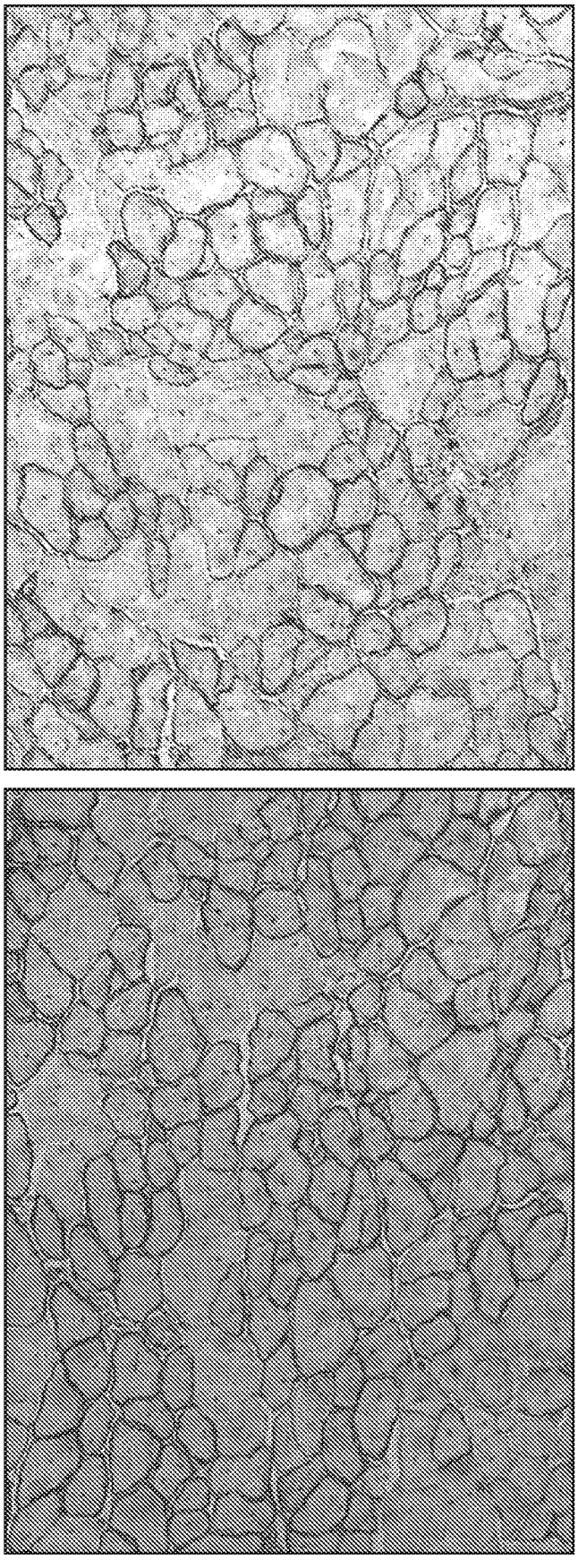


FIG. 9

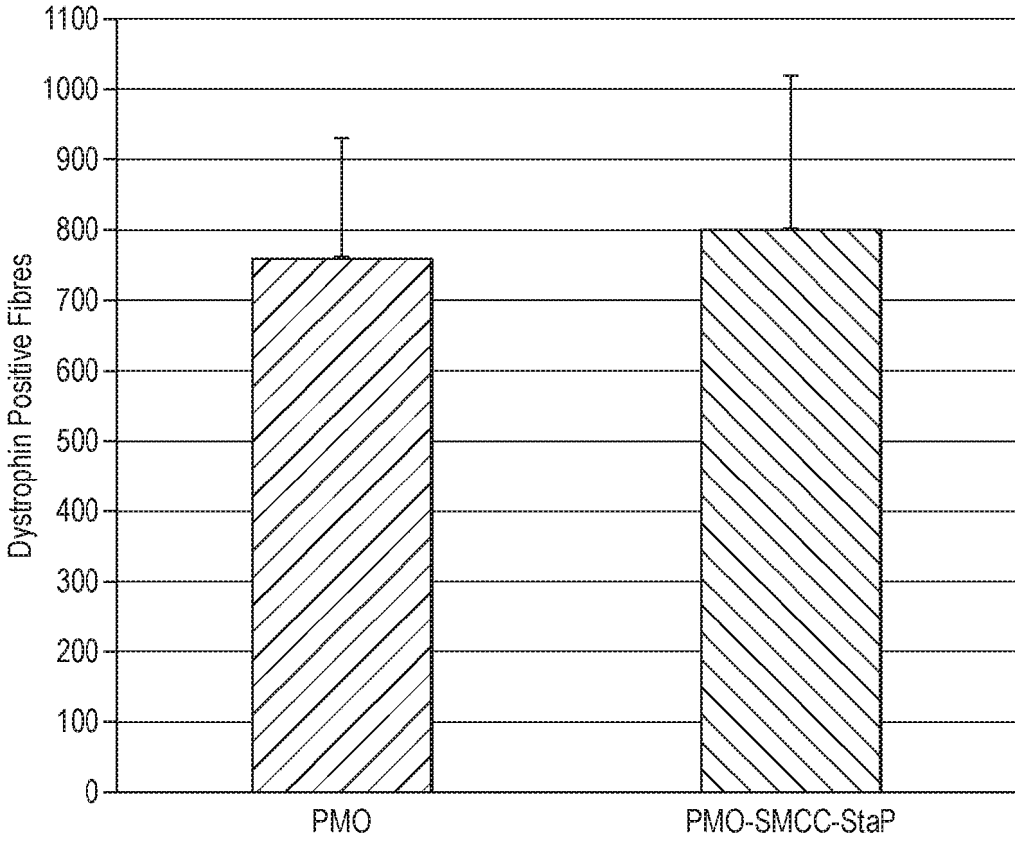


FIG. 10

21 03 17

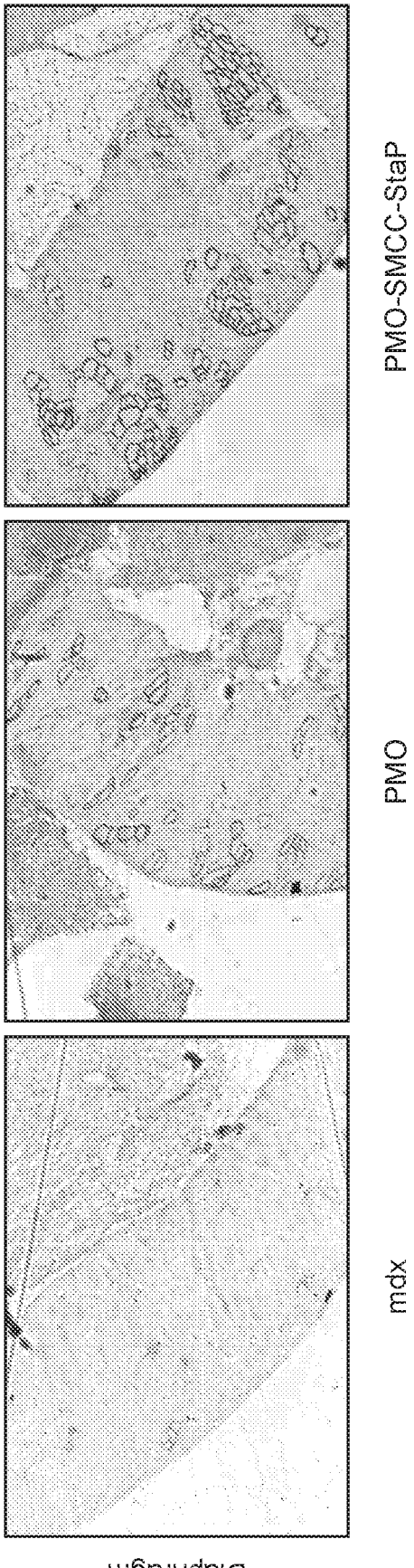


FIG. 11

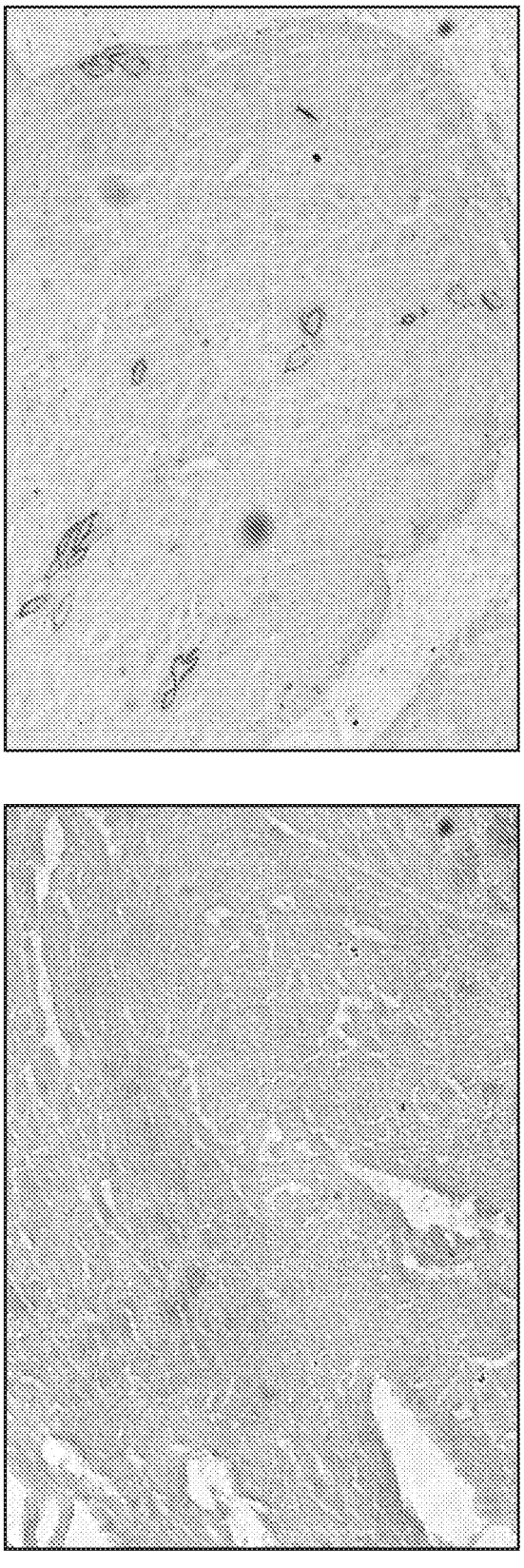


FIG. 12

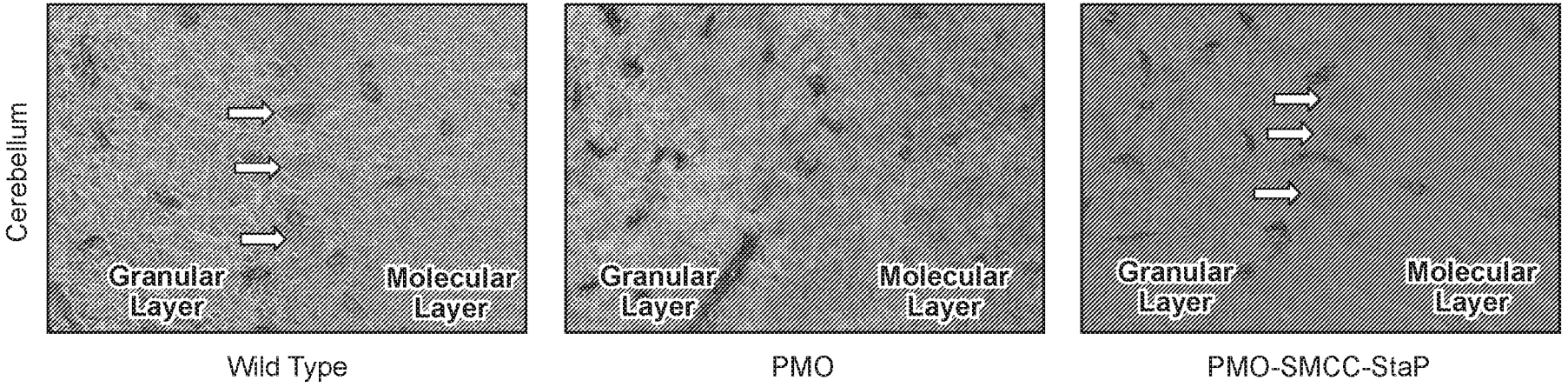

Cerebellum
Granular Layer
Molecular Layer
Granular Layer
Molecular Layer
Granular Layer
Molecular Layer
Wild Type
PMO
PMO-SMCC-StaP

FIG. 13

IMPROVED DRUG DELIVERY BY CONJUGATING OLIGONUCLEOTIDES TO STITCHED / STAPLED PEPTIDES

[0001] The present invention relates to improvements in drug delivery.

[0002] More particularly it relates to the use of Cell Penetrating Agents (CPA's), and more particularly still to the use of Cell Penetrating Peptides (CPP's) which have been stabilized by, for example: i) stapling two amino acids to form Stapled CPP's (StaP's) or ii) stitching three or more amino acids to form stitched CPP's (StiP's).

[0003] These stabilized CPP's are conjugated to a drug or Biologically Active Compound (BAC) directly or via a Bi-Functional Linker (BFL) so that the BAC can be carried though a cell membrane by the CPP. The resulting molecules are referred to as Drug Carrying Cell Penetrating Molecules (DCCPM's).

[0004] The preferred BAC's delivered in this manner are oligonucleotides (ON's).

[0005] The invention also relates to a method of facilitating the uptake of a BAC into a cell, the use of a DCCPM in the treatment of a disease requiring alteration of an endogenous or exogenous gene, a method of improving the bioavailability of a drug or BAC, a method of introducing a drug or BAC to a site which is refractory to the drug or BAC in its native state, a method of treating a subject comprising administering the DCCPM's of the invention and to a pharmaceutical composition comprising the DCCPM and one or more pharmaceutically acceptable excipients.

BACKGROUND TO THE INVENTION

[0006] In the treatment of all diseases it is desirable to deliver a drug or BAC into the body, and more preferably into a cell, at a target site, in a manner that ensures a maximal effect with minimal toxicity. This can be challenging.

[0007] An example of drugs or BACs which are delivered in a targeted manner are oligonucleotides (ON's), which term includes ON analogues.

[0008] ON's can target essential DNA and RNA sequences and can modulate gene expression in a number of ways that includes steric blocking to suppress (i) RNA splicing, (ii) protein translation or (iii) other nucleic acid:nucleic acid or nucleic acid:protein interactions.

[0009] Specifically, the hybridisation of ON's to specific RNA sequence motifs prevents correct assembly of the spliceosome, so that it is unable to recognise the target exon(s) in the pre-mRNA and hence excludes these exon in the mature gene transcript. Exclusion of an in-frame exon can lead to a truncated yet functional gene product; exclusion of an out of frame exon results in a frame-shift of the transcript, potentially leading to a premature stop codon and a reduction in the target gene expression level.

[0010] Additionally, ON's can be designed to target 5' translation initiation start sites of viral gene transcript(s) to prevent binding of the translational machinery. Using antisense oligonucleotides (ASO) to suppress viral translation is a well-established technology[1] and has progressed into clinical trials for viral haemorrhagic fevers such as Marburg and Ebola[2,3].

[0011] Also, ON's can be designed to form aptamers such that the secondary and tertiary structures can bind proteins or other cellular targets thus impacting on specific gene expression levels.

[0012] An advantage of steric blocking based suppression is that siRNA/RNAi based RNase H-induction of the RNA Induced Silencing Complex results in a reduced likelihood of off target side effects. In addition, ON's can be engineered as aptamers, to bind small proteins or metabolites.

[0013] Indeed, uncharged ON backbones such as phosphorodiamate morpholino oligonucleotides (PMOs) represent attractive BAC's and have an impeccable safety record in a preclinical and clinical setting [2,4-7]. However, their ability to penetrate cells and access their targets is compromised due to their uncharged nature [8]

[0014] Overcoming the problem of facilitating their entry into cells is therefore desirable.

[0015] Over the last 20 years much research has been dedicated to developing CPA's that facilitate delivery of drugs and BAC's to the biological site of action.

[0016] The approach has been to use charged peptides, either as non-covalent complexes or as covalent conjugates, to facilitate cell entry of a BAC. A peptide capable of effecting peptide-mediated cell delivery may also be referred to as a Cell Delivery Peptide (CDP). Examples include: poly arginine, penetratin (based upon an antennapedia homeodomain), or PMO internalization peptides (PIPs).

[0017] However, since their first description, [9] and given that many CPPs contain multiple arginines, β-alanine and 6-aminohexanoic acid residues, (*e.g.* poly-Arg12, TAT, Penetratin, Pip6a) [database maintained at http://crdd.osdd.net/raghava/cppsite/][10], it is surprising that no CPP-delivered drug has progressed through all phases of clinical trials. In part, this may be because the common arginine-rich core, which makes most CPP's effective, also causes membrane deformities [11] and in higher mammals that manifests as prohibitive toxic side effects such as tubular degeneration of the kidney [12].

[0018] The Applicant has overcome this major impediment by utilising stabilized CPA's. By linking a drug or BAC to a stabilized CPA, including stitched and stapled peptides, they have surprisingly obtained enhanced cellular uptake dynamics, 10-20 fold better than current state of the art CPA's[13,14].

[0019] One way to prepare stapled and stitched peptides, two linked amino acids (stapled) or three or more linked amino acids (stitched), is to incorporate amino acids into the peptide that are modified to bear *e.g.* an olefin (alkene) group (which may be incorporated at defined relative positions during solid-phase peptide synthesis). For example, on-resin ring-closing metathesis is

then used to close one (stapled) or two or more (stitched) all-hydrocarbon cross-links that induce the peptide to adopt a stabilised structure, typically, but not essentially an alpha helix. For StaP's, it is preferred to use either one or both enantiomers of the un-natural amino acids, termed the **S5** (*S*-pentenylalanine) or **R5** (*R*-pentenylalanine), or the **S8** (*S*-octenylalanine) or **R8** (*R*-octenylalanine), depending on the stereo-chemical configuration. For StiP's, a further un-natural olefin-bearing α, α-di-substituted amino acid (**B5** or **B8**) is utilised. Cross linking strategies are however not restricted to ring-closing metathesis of un-natural olefin-bearing α, α-di-substituted amino acids. Other cross-linking chemistry's may be used to stabilize the peptide, such as ring-closing metathesis between O-allylserine analogues (**S-OAS** or **R-OAS**).

[0020] The cellular entry dynamics of existing CPAs and the StiP's and StaP's differ. Traditional CPPs enter cells via energy-independent direct plasma membrane translocation or via energy-dependent, clathrin and caveolin-mediated endocytosis; whereas the StiP's and StaP's utilised in the invention enter via an energy dependent, but clathrin and caveolin independent mechanism [13,15]. Given that StiP's and StaP's uptake is abrogated with reduced cellular decoration of heparin sulphate [13] a macropinocytotic entry mechanism is inferred[16].

[0021] Relative to their unmodified peptide precursors, all-hydrocarbon StaP's and StiP's generally exhibit robust cellular uptake, significant resistance to proteolytic degradation, and *in vivo* stability that can support a half-life of more than 12 hours in non-human primates[17]. It is likely that this increase in drug-likeness stems from the highly rigidified structure and the burial of the backbone amide bonds in the core of *e.g.* the α-helix. This structural rigidity also decreases the likelihood that StiP's and StaP's will be immunogenic, as the design of major histocompatibility complexes is such that peptides must adopt an extended conformation to be presented. The potential lack of membrane toxicity and immunogenicity enhances the clinical translatability of compounds when conjugated to drugs and BAC's such as ON's.

[0022] ONs can be used to induce a steric block to any gene in humans, animals and lower order organisms and thus can be applied to natural disease (including genetic and age-related diseases) or acquired diseases in humans and animals.

[0023] For example, viral haemorrhagic fevers (VHFs) are animal-borne illnesses in which a prolonged inflammatory cytokine response leads to the gradual destruction of veins and arteries. Causes of VHF include Ebola and Marburg viruses and several Arenaviruses; these diseases are presently considered untreatable. Viral haemorrhagic fevers are characterized by high fever and bleeding disorders, and can cause death by shock and organ failure. ASOs can be designed to target 5' translation initiation start sites of viral gene transcript(s) to prevent binding of the translational machinery. Using ASO to suppress viral translation is a well-established technology [1] and has progressed into clinical trials for viral haemorrhagic fevers such as Marburg and Ebola [2,3]. One PMO, AVI-7537 was evaluated for human use in the West African Ebola outbreak in 2014-15.

[0024] Some tissues are particularly refractory to naked PMO transfection, *e.g.* heart, which may reflect differential vesicle-mediated PMO uptake mechanisms [15]. In fact, direct intra-cardiac injection of naked PMO does not even lead to efficient transfection [18], and refractory tissues tend to require repeat administration or high dose strategies [19-21]. However, whilst CPP conjugation improves PMO bio-distribution and serum stability[22-24], toxicity is still a major roadblock for pipeline development[12].

[0025] For effective clinical translation of steric blocking ASOs, CPPs need to effectively deliver the BAC to either the cytoplasm or nucleoplasm whilst limiting any toxicity associated with cell entry.

[0026] Thus, providing DCCPM's which are able to deliver a drug or BAC more efficiently or to a target site, or with lower toxicity would be highly desirable.

BRIEF SUMMARY OF THE DISCLOSURE

[0027] In accordance with a first aspect of the present invention there is provided a drug carrying cell penetrating molecule (DCCPM) comprising:

i. a biologically active compound (BAC), and
ii. a cell penetrating agent (CPA), which BAC and CPA are linked directly or via a bi-functional linker (BFL),

and wherein the CPA is a stabilized peptide (CPP) which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP), the StaP comprises a cross link or bridge between two conformationally adjacent amino acids of the peptide and the StiP comprises a cross link or bridge between at least three conformationally adjacent amino acids of the peptides and the BAC is an electrically neutral oligonucleotide (charge -1 to +1 at pH 7.5) (ON).

[0028] In a preferred embodiment the cross link or bridge comprises two components, a hydrocarbon bridge and a terminal methyl group.

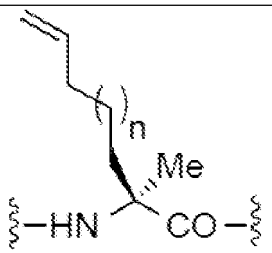
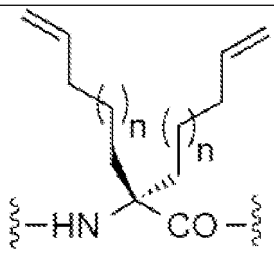
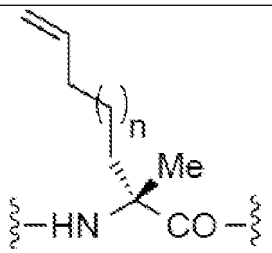
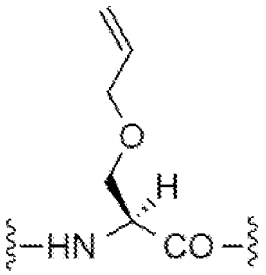
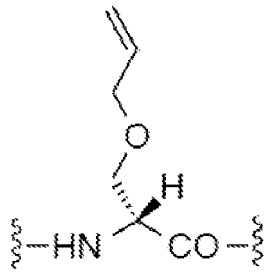
[0029] The hydrocarbon bridge may be composed of a double hydrocarbon bond or following a further reduction to a single hydrocarbon bond.

[0030] The CPP preferably comprises at least two un-natural amino acids bearing all-hydrocarbon tethers (*e.g.* α-methyl,α-pentenyl glycine).

[0031] The preferred stapled or stitched CPPs incorporate one or more of: a (*S*)-pentenylalanine (**S5**) or its enantiomer (**R5**), a *S*-octenylalanine (**S8**) or its enantiomer (**R8**) or combinations thereof (e.g *R*-octenylalanine/*S*-pentenylalanine (**R8**/**S5**) or *S*-octenylalanine/*R*-pentenylalanine (**S8**/**R5**).

[0032] The preferred unnatural amino acids incorporated into the CPPs and reacted to form a cross link or bridge between them are illustrated in Table 1 and some exemplary and preferred resulting CPPs are illustrated in Table 2.

[0033] Table 1

Unnatural Amino Acids		
 **S5** : n=1 **S8** : n=4	 **B5** : n=1 **B8** : n=4	 **R5** : n=1 **R8** : n=4
 **S-OAS**	 **R-OAS**	

[0034] Table 2

Peptide sequence	Length	Peptide Type
βAla-RKF-S5-RLF-S5	8	i, i+4 Staple
βAla-RKF-S5-RLF-S5 (reduced)	8	i, i+4 Staple
βAla-RKF-S5-RLF-S5-SY	10	i, i+4 Staple
βAla-RKF-S5-RLF-S5-SY (reduced)	10	i, i+4 Staple
βAla-RELRREI-S5-LCR-S5-HHST	16	i, i+4 Staple
βAla-RELRREI-S5-LCR-S5-HHST	16	i, i+4 Staple
βAla-S8-RQARRN-B5-RRRWRE-S8-QR	17	i, i+4, i+11 Stitch
βAla-S8-RQARRN-B5-RRRWRE-S8-QR (reduced)	17	i, i+4, i+11 Stitch
βAla-S8-RQARRQ-B5-RRRWRE-S8-QR	17	i, i+4, i+11 Stitch
βAla-S8-RQARRQ-B5-RRRWRE-S8-QR (reduced)	17	i, i+4, i+11 Stitch
βAla-TRQ-S5-RRN-B5-RRRWRE-S8-QR	17	i, i+4, i+11 Stitch
βAla-TRQ-S5-RRN-B5-RRRWRE-S8-QR (reduced)	17	i, i+4, i+11 Stitch
βAla-TRQ-S5-RRQ-B5-RRRWRE-S8-QR	17	i, i+4, i+11 Stitch
βAla-TRQ-S5-RRQ-B5-RRRWRE-S8-QR (reduced)	17	i, i+4, i+11 Stitch
βAla-TRQ-S5-RRA-B5-RRRWRE-S8-QR	17	i, i+4, i+11 Stitch
βAla-TRQ-S5-RRA-B5-RRRWRE-S8-QR (reduced)	17	i, i+4, i+11 Stitch

S5= α-methyl, α-alkenylglycine with 5 carbon chain
S8= α-methyl, α-alkenylglycine with 8 carbon chain
B5= α-methyl, α-alkenylglycine with two 5 carbon chain

[0035] Alternative CPPs and their method of manufacture are disclosed in Chu et al, 2014 and associated supplementary information and are incorporated by reference[13].

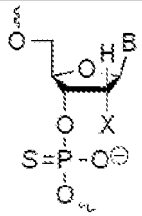
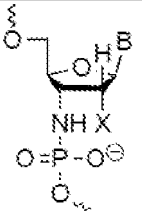
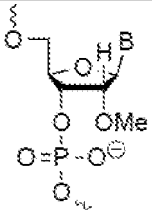
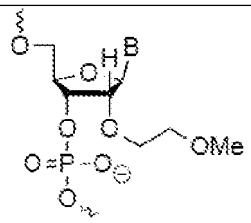
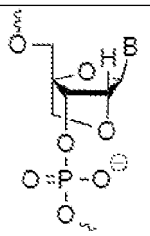
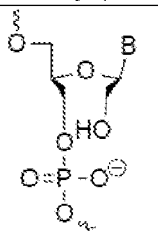
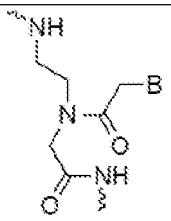
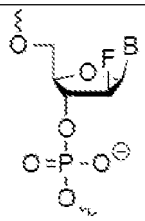
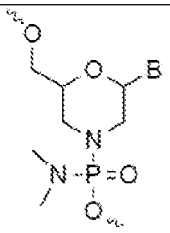
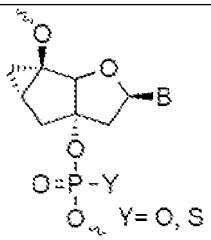
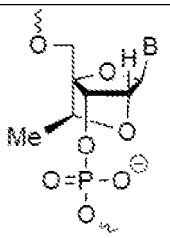
[0036] The exemplified stabilized peptide comprises two or more olefin bearing side chains that are covalently formed, typically by means of a ring-closing metathesis.

[0037] The stabilized conformation typically comprises at least one alpha helix. It may however, in the alternative, comprise at least one beta sheet or comprises at least one alpha helix and one beta sheet.

[0038] The preferred BAC is an oligonucleotide (ON), more preferably still an anti-sense oligonucleotide (AON).

[0039] Different anti-sense oligonucleotide chemistries are illustrated in Table 3 with the use of phosphorodiamate morpholino oligonucleotides (PMOs) being preferred.

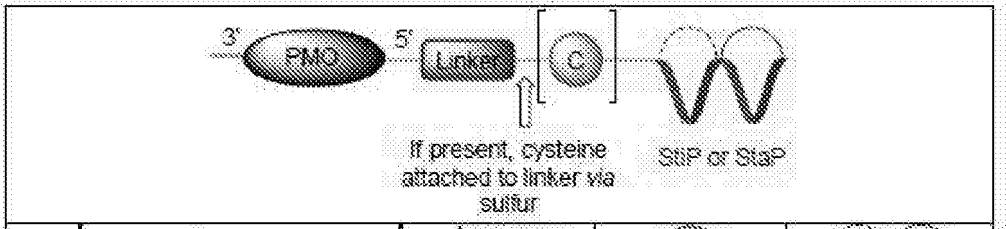
[0040] Table 3

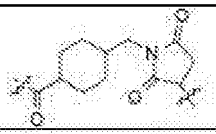
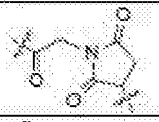
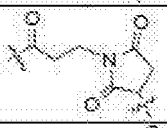
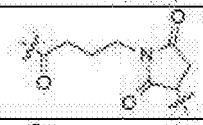
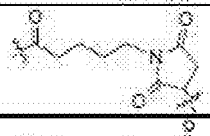
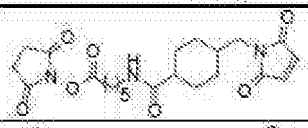
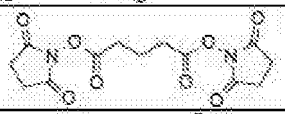
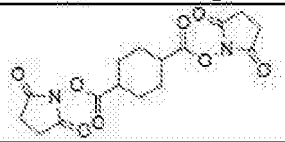
 Phosphorothicate (**PS**)	 N'3 Phosphoramidite (**NP**)	 2'-*O*-Methyl (**2' O-Me**)
 2'-*O*-Methoxyethyl (**MOE**)	 Locked Nucleic Acid (**LNA**)	 Unlocked Nucleic Acid (**UNA**)
 Peptide Nucleic Acid (**PNA**)	 2'F-Arabino Nucleic Acid (**2'F-ANA**)	 Phoshoramidate morpholino (**PMO**)
 Tricyclo-DNA (**tcDNA**)	 S-constrained-ethyl (**cET**)	

[0041] The BAC may target and alter the expression of an endogenous or exogenous gene. Endogenous gene targets include but are not limited to genes associated with neuromuscular disease, metabolic disease, cancer, age-related degenerative diseases and exogenous gene targets include those of an acquired viral infection.

[0042] Whilst the BAC may be linked to the CPP directly the Applicant has found the use of a desirable BFL. Exemplary, non-limiting BFL chemistries are illustrated in Table 4.

[0043] Table 4



Entry	Linker	Acronym for linker reagent	C Requirement	
1		SMCC	Y	e.g. βAla-RKF-S5-RLF-S5
2		AMAS	Y	
3		BMPS	Y	
4		GMBS	Y	
5		DMVS	Y	
6		EMCS	Y	
7		LC-SMCC	Y	
8		DSG	N	
9		DSCDS	N	

[0044] In a preferred chemistry an amine to sulphydryl cross linker containing N-hydroxysuccinimide esters and malemide reactive groups separated by a cyclohexane spacer is utilized, namely succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC – Compound II, Fig 1).

[0045] Thus a CPA, such as Compound III (Fig 1) may be covalently linked to a first end of the BFL (Compound II) with the BAC (Compound I) being covalently or non-covalently linked to a second end of the BFL to generate a DCCPM (Compound V).

[0046] According to a second aspect of the invention there is provided a method for facilitating the uptake of a biologically active compound (BAC), which is is an electrically neutral oligonucleotide (charge -1 to +1 at pH 7.5) (ON), into a cell by the conjugation of the

oligonucleotide (ON) to a cell penetrating agent (CPA) which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP), the StaP comprises a cross link or bridge between two conformationally adjacent amino acids of the peptide and the StiP comprises a cross link or bridge between at least three conformationally adjacent amino acids of the peptides directly or via a bi-functional linker (BFL) to form a drug carrying cell penetrating molecule (DCCPM) and presenting said DCCPM to said cell in a suitable vehicle.

[0047] According to a third aspect of the present invention there is provided a DCCPM of the first aspect of the invention for use in the treatment of a disease requiring alteration of the expression of an endogenous or exogenous gene.

[0048] The DCCPM may be used in the treatment of a, for example, neuromuscular disease, metabolic disease, cancer, age-related degenerative disease or to treat an acquired viral infection.

[0049] In one embodiment the DCCPM is used in the treatment of a muscular dystrophy e.g. Duchenne muscular dystrophy.

[0050] In such an embodiment the DCCPM may comprise an AON targeting exon 51 of the dystrophin gene.

[0051] In accordance with a forth aspect of the present invention there is provided a method of improving the bioavailability of a drug or biologically active compound (BAC), which is is an electrically neutral (charge -1 to +1 at pH 7.5) oligonucleotide (ON), comprising linking the ON to a stabilized peptide (CPP) which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP), the StaP comprises a cross link or bridge between two conformationally adjacent amino acids of the peptide and the StiP comprises a cross link or bridge between at least three conformationally adjacent amino acids of the peptides.

[0052] In accordance with a fifth aspect of the present invention there is provided a method of introducing a drug or BAC to a site which is refractory to a drug or BAC in its native state comprising linking the drug or BAC to a CPP which is a stabilized peptide and administering it to a subject.

[0053] The DCCPMs of the invention can be used to administer the drug or BAC to a target tissue, such as, for example the heart, brain or muscle.

[0054] In accordance with a sixth aspect of the present invention there is provided a method of treating a subject to alter the expression of an endogenous or exogenous gene comprising administering a DCCPM of the invention to a subject.

[0055] In accordance with a seventh aspect of the present invention there is provided a composition comprising a DCCPM of the invention and one or more pharmaceutically

acceptable excipients enabling the composition to be administered orally, parenterally, intravenously or topically.

BRIEF DESCRIPTION OF THE DRAWINGS

[0056] Embodiments of the invention are further described hereinafter with reference to the accompanying Drawings, in which:

[0057] Figure 1 shows a general schematic of a FITC labeled DCCPM (compound V). A FITC labeled PMO (compound I) is linked to succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC; compound II) forming a SMCC linked PMO intermediate (compound IV) and then to a CPA (compound III) , forming compound V.

[0058] Figure 2 shows a general schematic of a FITC labeled DCCPM in which the n-termini of the FITC labelled PMO (compound I) and the CPA (compound III) are linked via a bi-functional linker disuccinimidyl glutarate (DSG) forming compound VI.

[0059] Figure 3 shows a general schematic of a FITC labeled DCCPM in which the FITC labelled PMO (compound I) and the CPA (compound III) are linked directly forming compound VII.

[0060] Figure 4 shows the liquid chromatography-mass spectrometry conformation of the synthetic steps and the molecular masses of compound I (Figure 4a); compound IV (Figure 4b); and compound V (Figure 4c).

[0061] Figure 5 shows a dose dependent increase in DCCPM delivery into a human osteosarcoma cell line (U2OS) maintained in culture, without transfection reagent. The biologically active compound was a PMO with a sequence:

Sequence id 1: 5'GGCCAAACCTCGGCTTACCTGAAAT3'

(an antisense reagent targeted to exon 23 of the mouse dystrophin gene, that causes the exclusion of exon 23 during mRNA splicing maturation); the bi-functional linker was SMCC; the StaP was βAla-RKF-S5-RLF-S5. This configuration of DCCPM is depicted as PMO-SMCC-StaP in the subsequent figures.

[0062] Figure 6 shows a graphical representation of the dose dependent increase in PMO-SMCC-StaP delivery into U2OS cells maintained in culture, without transfection reagent shown in Figure 5.

[0063] Figure 7 shows PMO-SMCC-StaP delivery into a mouse cell line that harbours the *mdx* mutation of the dystrophin gene (H2K *mdx*) maintained in culture, without transfection reagent.

[0064] Figure 8 shows an agarose gel electrophoresis image demonstrating that exon exclusion of the mouse dystrophin exon 23 is restricted to H2K *mdx* cells that have been transfected with

either 5μM PMO-SMCC-StaP or 5μM naked PMO alone. 24 hours after incubation with PMO-SMCC-StaP or naked PMO, H2K *mdx* cells were recovered and RNA isolated. The RNA was reversed transcribed and an amplification between exons 20 and 26 of the mouse dystrophin gene, followed by a nested amplification between exon 20 and 26 was performed to yield a full length product of 901 bp fragment if exon 23 is present or 688bp fragment if exon 23 is excluded.

[0065] Figure 9 shows immunocytochemical staining for dystrophin from *Tibialis anterior* muscles of *mdx* mice following an intramuscular injection of either 2.2 nmol PMO-SMCC-StaP or 2.2nmol naked PMO into the *Tibialis anterior* muscle. Muscles were recovered 7 day post administration.

[0066] Figure 10 shows a graphical representation of an immunocytochemical staining for dystrophin from *Tibialis anterior* muscles of *mdx* mice injected with either 2.2 nmol PMO-SMCC-StaP or 2.2nmol naked PMO. The total number of dystrophin positive fibres is equivocal between groups, with no statically significant difference. This data suggest that the conjugation of a SMCC-StaP complex to a PMO does not alter the biological activity of the PMO.

[0067] Figure 11 shows immunocytochemical staining for dystrophin from diaphragm muscles of *mdx* mice following an intraperitoneal injection of 9mg/kg PMO-SMCC-StaP or 9mg/kg naked PMO. Muscles were recovered 7 days post administration. There is a greater level of dystrophin restoration in diaphragm muscles from PMO-SMCC-StaP treated cohort compared to the PMO cohort.

[0068] Figure 12 shows immunocytochemical staining for dystrophin from heart muscles of mdx mice following an intraperitoneal injection of 9mg/kg PMO-SMCC-StaP or 9mg/kg naked PMO. Muscles were recovered 7 days post administration. Dystrophin restoration was restricted to heart muscle of the PMO-SMCC-StaP treated cohort only. The heart is refractory to PMO induced restoration of dystrophin at this dose.

[0069] Figure 13 shows immunocytochemical staining for dystrophin from the cerebellum of mdx mice following an intraperitoneal injection of 9mg/kg PMO-SMCC-StaP or 9mg/kg naked PMO. Cerebella were recovered 7 days post administration. Dystrophin restoration was restricted to purkinje cell of the PMO-SMCC-StaP treated cohort only. The cerebellum is refractory to PMO induced restoration of dystrophin at this dose.

DETAILED DESCRIPTION

[0070] The invention is illustrated with reference to a single example which proves the benefit of the claimed invention.

[0071] An exemplary drug carrying cell penetrating molecule (DCCPM) was produced with a FITC label in order to demonstrate cellular uptake (Example 1).

[0072] The exemplary DCCPM comprises:

i) a biologically active compound (BAC) – (see Table 3 for non-limiting examples);

ii) a cell penetrating agent (CPA) which is a stabilized peptide (See Table 1 for non-limiting examples); and

iii) a bi-functional linker (BFL) (see Table 4 for non-limiting examples)

[0073] The three components forming the DCCPM are described in more detail below, although as illustrated in Fig 3, the BAC and CPA can be linked directly.

1. **The Biologically Active Compound**.

[0074] The biologically active compound is any compound that can exert a biological effect within a biological cell. Preferably, though not essentially, the BAC is one which will impact on the expression of one or more endogenous or exogenous genes. Examples include nucleic acids, DNAzymes, ribozymes, aptamers and pharmaceuticals. Preferred biologically active compounds for use in the present invention include electrically neutral oligonucleotides (charge -1 to +1 at pH 7.5) such as polynucleic acids (PNAs) or PMOs or their modified derivatives that might impart an electric charge (either positive or negative).

[0075] The biologically active compound may be used as a steric blocking compound to suppress or enhance: i) RNA splicing; ii) protein translation or iii) other nucleic acid:nucleic acid or nucleic acid:protein interactions, altering the gene expression of endogenous or exogenous (pathogen derived) genes.

[0076] The hybridisation of ON's to specific RNA sequence motifs prevents correct assembly of the spliceosome, so that it is unable to recognise the target exon(s) in the pre-mRNA and hence excludes these exon in the mature gene transcript. Exclusion of an inframe exon can lead to a truncated yet functional gene product; exclusion of an out of frame exon results in a frame-shift of the transcript, potentially leading to a premature stop codon and a reduction in the target gene expression level.

[0077] Additionally, ON's can be designed to target 5' translation initiation start sites of viral gene transcript(s) to prevent binding of the translational machinery. Using ASO to suppress viral translation is a well-established technology and has progressed into clinical trials for viral haemorrhagic fevers such as Marburg and Ebola.

[0078] Also, ON can be designed to form aptamers such that the secondary and tertiary structures can bind proteins or other cellular targets thus impacting on specific gene expression levels.

[0079] Non-limiting exemplary ON chemistries are illustrated in Table 3.

[0080] In one example, the target is exon 51 of the dystrophin gene and comprises the sequence:

Sequence id 2: 5'CUCCAACAUCAAGGAAGAUGGCAUUUCUAG3'

2. The cell penetrating agent (CPA) which is a stabilized peptide

[0081] The cell penetrating agents of the invention are stabilized peptides.

[0082] The peptides may be stabilized by stapling, to form a stapled peptide (StaP), or by stitching to form a stitched peptide (StiP)

[0083] All-hydrocarbon staples and stitches may confer, on *e.g.* an α-helical structure, protease resistance, cellular penetrance, and biological activity.

[0084] Non-limiting examples of stapled and stitched peptide sequences are illustrated in Table 2 and include peptide sequences including S5, S8 and B5 (as defined in Table 2).

[0085] Stabilisation of *e.g.* the α-helical structure can be achieved by, for example, a ring-closing metathesis and may be catalysed by a variety of ruthenium catalysts including Grubbs generations 1 and 2 and Grubbs-Hoyveda generations 1 and 2.

[0086] All the peptide components (amino acids, unnatural amino acids, unstapled/unstitched, partially stapled/stitched and stapled/stitched peptides) may exist in specific geometric or stereoisomeric forms. All compounds include *cis*- and *trans*-isomers, (R)- and (S)-enantiomers, diastereoisomers and racemic mixtures thereof.

[0087] Preferred isomer/enantiomers will be enriched to give a greater proportion of one particular isomer/enantiomers. Embodiments thereof may be made of 90%, 95%, 98% or 99% by weight of a preferred isomer/enantiomers.

[0088] Non-limiting examples of unnatural amino acids used in stabilising a peptide structure are illustrated in table 1.

[0089] In one embodiment the applicant employs α,α-disubstituted unnatural amino acids bearing all-hydrocarbon tethers (*e.g.* α-methyl,α-pentenyl glycine)

[0090] For single turn stapling, one embodiment could employ a (*S)*-pentenylalanine (**S5**) at, *e.g. i*, *i* + 4 positions, and in another embodiment, for double turn stapling, a combination of either *R*-octenylalanine/*S*-pentenylalanine (**R8**/**S5**) or *S*-octenylalanine/*R*-pentenylalanine (**S8**/**R5**) at *e.g. i*, *i* + 7 positions can be used. The same pairings can be used to install more than one staple within a given peptide template. **S5** can be substituted at *i*, **B5** at postion *i* + 4 positions, and **S8** can be been substituted at *i*, *i* +4, *i* + 11 positions to generate stitched peptides. The **S5** configured amino acid and its enantiomer R5, or S8 configured amino acid and its enantiomer **R8**, differ only in the opposite stereochemical configuration of the staple they bear.

[0091] Based upon the inclusion of a single or a double turn staple peptides may comprise of one or more of the sequences in Table 2. Based upon specific peptide shown in Table 2, a person skilled in the art can easily envisage peptides with 3, 4, 5 or more turn stabilising staples.

[0092] In one embodiment the cell penetrating agent has a stitch or staple peptide comprising the sequence β-Ala-RFK-S5-RLF-S5.

[0093] In another embodiment the peptide is a branched stapled peptide. The branched stapled peptide comprises of 2 or more chains of peptides. Branched peptides may be formed using any method know to the art; in one embodiment a lysine residue is used to branch two peptide chains.

[0094] Functional derivatives of disclosed peptide sequences could be used. Functional derivatives may have representative fragments or homologues or peptides that include insertions to the original peptide. Typical derivative would have 70%, 80%, 90% or more of the original peptide sequence and may have up to 200% of the number of amino acids of the original peptide. The derivatives would be used to enhance the delivery of a biologically active compounds.

3. Bi-functional Linker

[0095] A bi-functional linker may be used to link the BAC to the CPA.

[0096] Preferred linkers will link between, for example, an amine group and a sulfhydryl (thiol) group (usually a cysteine residue). Examples of substrates to achieve this include, but are not limited to, SMCC (succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate), AMAS (N-α-maleimidoacet-oxysuccinimide ester, BMPS (N-β-maleimidopropyl-oxysuccinimide ester), GMBS (N-γ-aleimidobutyryl-oxysuccinimide ester), DMVS (N-δ-maleimidovaleryl-oxysuccinimide ester, EMCS (N- ε-malemidocaproyl-oxysuccinimide ester), and LC-SMCC (Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxy-(6-amidocaproate) as exemplified in Table 4.

[0097] Other linkers such as DSG (disuccinimidyl gluterate) and DSCDS (disuccinimidyl-cyclohexl-1,4-diester) will include the ability to link the 5'-amino group of the BAC to the N-terminus of the CPA (Table 4, entries 8 and 9).

[0098] The biologically active compound is covalently or non-covalently attached to the chimeric cell delivery peptide. Again, this can be done using any method known in the art. Preferably, the cell delivery peptide is attached to the biologically active compound by means of a disulphide bridge or a thiol maleimide linker *e.g.* SMCC; the attachment may be by means of an amide linker (preferably a stable amide linker) or an oxime linker or a thioether linker.

EXAMPLE 1: (Proof of principle)

DCCPM to enhance RNA steric blocking in treating Duchenne muscular dystrophy (DMD).

Introduction

[0099] Duchenne muscular dystrophy (DMD) is the most common inherited lethal childhood disease in the world, with a worldwide incidence of approximately 1 in 4000 live births [25]. This severe muscle-wasting disorder is caused in the majority of families by gene mutations leading to disruption of the reading frame and premature truncation of the protein dystrophin [26,27].

[00100] RNA splicing suppression of the DMD transcript has particular promise. The hybridisation of ASOs to specific RNA sequence motifs prevents correct assembly of the spliceosome, so that it is unable to recognise the target exon(s) in the pre-mRNA and hence excludes them in the mature gene transcript. ASO-mediated RNA splicing suppression resulting in the re-expression of a truncated, yet functional dystrophin protein has been demonstrated *in vitro* and in the pre-clinical *mdx* mouse model[21,28-33], which led to clinical development programs[7,34].

[00101] Although intravenously administered PMOs have demonstrated a dose-dependent increase in dystrophin re-expression with some functional benefit[7,35], skeletal muscle dystrophin restoration is still very variable between patients after many multiple administrations. Importantly, many other target tissues (*e.g.* brain and heart) remain refractory to PMO transfection even when repeat administration or high dose strategies are employed[19-21].

[00102] To date unmodified CPA conjugation improves PMO bio-distribution and serum stability[22-24], however toxicity is still a major roadblock for pipeline development[12].

[00103] Applicant hypothesised that a CPA based upon a stabilized *e.g.* StaP (or StiP) conjugated to a PMO known to cause RNA splicing suppression of the DMD transcript, would lead to a greater level of dystrophin restoration and re-expression of dystrophin in tissues refractory to naked PMO without the potential for CPA related toxicity.

Materials and Methods

PMO and Peptide Conjugation

PMO were synthesised with a 5' amine group and 3' fluorescein isothiocyanate (FITC) label and purified >90% by Genetool LLC (Philomath, Oreg. USA). All peptides were synthesized

following an established protocol using standard Fmoc-peptide chemistry on Rink amide MBHA resin. The coupling reactions were performed by the addition of a mixture of 10 equivalents of the amino acids, 9.9 equivalents of HCTU and 20 equivalents of DIPEA in NMP (equivalents relative to initial loading of Rink amide MBHA resin). The reactions were allowed to proceed for at least one hour. Coupling of non-natural amino acids (*R*/*S*5, *R*/*S*8 or *B*5) was performed with 4 equivalents of the amino acid, 3.9 equivalents of HCTU and 10 equivalents of DIPEA in NMP for two hours. The ring closing metathesis reaction of the olefin-containing non-natural amino acids was facilitated with Grubbs I catalyst (benzylidene-bis(tricyclohexylphosphine)-dichlororuthenium) dissolved to approximately 10mg/mL in 1,2-dichloroethane (DCE) for two hours under nitrogen bubbling. Subsequently, excess catalyst was washed from the resin with DCE and then coupled with an N-terminal FITC. Upon completion, peptides were simultaneously cleaved from the resin and de-protected using a cleavage cocktail containing 95% TFA, 2.5% TIS and 2.5% water. Crude peptides were dissolved in 50% acetonitrile/water, passed through a 0.2 µm syringe filter, and purified by reverse phase HPLC using a C-18 column (Agilent, Palo Alto, CA). Compound identification and purity was assessed using coupled LC/MS (Agilent, Palo Alto, CA). Purified fractions were pooled and evaporated to remove acetonitrile and trace TFA by Speedvac and then lyophilized to dryness.

Cell Culture and Transfection

[00104] U2OS cell (Human osteosarcoma) were cultured in high glucose DMEM supplemented with 10% foetal calf serum (Sigma, UK) at 37°c under a 8% CO2/92% air atmosphere.

[00105] H_2K mdx mouse myoblasts were cultured at 33°C under a 8% CO_2/92% air atmosphere in high-glucose DMEM supplemented with 20% fetal calf serum, 0.5% chicken embryo extract (PAA laboratories Ltd, Yeovil, UK), and 20 units/ml γ-interferon (Roche applied science, Penzberg, Germany). Cells were then treated with trypsin and plated at $8x10^4/cm^2$ in 24-well plates coated with 0.1mg/ml ECM gel (Sigma). H_2K mdx cells were transfected 24 h seeding with treatment in a final volume of 0.2 ml of normal growth media. Following 4 hours of transfection, the PMO or PMO-SAP was removed and replaced with DMEM supplemented with 5% horse serum. Fluorescence and RNA extraction was performed 48 hours post transfection.

[00106] U2OS cells were incubated with PMO or PMO-SMCC-StaP at increasing concentration (0.5µM, 1.0µM, 5.0µM and 10µM) with any facilitation transfection reagent. H_2K mdx mouse myoblasts were incubated with PMO or PMO-SAP at 5.0µM. Levels of FITC fluorescence was quantified at 494 nm to determine relative entry of PMO and PMO-SAP.

RNA Extraction and nested RT-PCR Analysis

[00107] Total RNA was isolated from H_2K mdx mouse myoblasts cells (RNeasy, Qiagen, UK). The RNA was reversed transcribed (nanoscript2, Primer Design UK) and an amplification between exons 20 and 26, followed by a nested amplification between exon 20 and 26 was performed to yield a full length product of 901 bp or 688bp if the mouse dystrophin exon 23 was excluded. Products loaded in a 1% agarose gel (buffered with tris acetate 40mM and 1mM ethylenediaminetetraacetic acid).

Animals

[00108] 12 week old mdx female mice were used in all experiments (n=4 for each control and test groups). All experiments were carried out in the Animal unit, School of Biological Science, University of Reading, Reading, UK according to procedures authorized by the UK Home Office. Mice were killed by CO_2 inhalation or cervical dislocation at desired time points, and muscles and other tissues were snap-frozen in liquid nitrogen-cooled isopentane and stored at –80°C.

Administration of PMO or PMO-StaCPA

[00109] Intramuscular administrations: Tibialis anterior muscles of mdx mice injected with either 2.2 nmol PMO-SMCC-StaP or 2.2nmol naked PMO. Systemic administration: mdx mice were subject to an intraperitoneal injection of 9mg/kg PMO-SMCC-StaP or 9mg/kg naked PMO.

Histology and Immunoctyochemistry

[00110] 10μM cryosections were cut and dystrophin protein was determined using rabbit polyclonal antibody to dystrophin (ab15277; Abcam, Cambridge, UK). Routine haematoxylin and eosin staining was used to assess general pathology and morphology.

Statistical Analysis

[00111] All data are reported as mean values ±SEM. Statistical differences between treatment groups and control groups were evaluated by SigmaStat (Systat Software, UK) and student's t test was applied. Significance was accepted for p-values<0.05.

Results

[00112] FITC labelled PMO was conjugated to the bi-functional linker (SMCC) and a StaP (βAla-RKF-S5-RLF-S5) as confirmed by mass spectrometry (Figure 4). Subsequently PMO-SMCC-StaP and naked PMO were transfected into a standard cell line (U2OS) to determine if the StaP conferred enhanced cell entry to the cell. Naked PMO were refractory to cell entry, giving only a background FITC fluorescence signal, compared to a dose dependant increase in fluorescence with PMO-SMCC-StaP (Figures 5 and 6). The lack of signal above background does not allow statistical analyses of the comparative increase in fluorescence, but clearly demonstrated that without the StaP conjugation, PMO did not enter the cell.

[00113] When transfection experiments were conducted in the H_2K mdx mouse myoblasts cells, it confirmed the finding that naked PMO are refractory to cell entry, which was overcome with the conjugation of a StaP (Figure 7) and that the StaCPA mediated delivery of PMO resulted in the steric blockade of RNA editing of the dystrophin transcript, such that exon 23 was excluded from the transcript (Figure 8).

[00114] In order to determine if the StaP hindered the biological activity of the PMO, direct intramuscular administrations (2.2 nmol) were conducted into the *Tibialis anterior* muscle of *mdx* female mice, with muscle recovered 7 days post-administration. The percentage of dystrophin re-expression was equivocal between the PMO-SMCC-StaP (805.75) and the naked PMO (762.25) with no statistical significant difference (n=4, p=0.863; Figures 9 and 10). Thus it was determined that the StaP does not confer any steric hindrance to the biological activity of the PMO.

[00115] Systemic intra-peritoneal administrations of PMO-SMCC-StaP and naked PMO were conducted in mdx females to determine if the SAP moiety enhanced cell entry. Sub-optimal amounts (9mg/kg) were administered and a series of tissues (skeletal muscle, heart and brain) recovered 7 days post-administration (Figures 11, 12 and 13).

Conclusion

[00116] From the example it can be seen that the conjugation of a CPA, stabilized by stapling, to a BAC (in the form of a PMO), via a BFL, facilitates entry of the PMO entry into a cell. The StaP CPA facilitated PMO entry in both *in vitro* and *in vivo* assay systems.

[00117] In the *in vivo* model of RNA splicing suppression demonstrated that the biological action of an α-helical peptide conjugated PMO is equivalent to naked PMO following intramuscular administration, thus determining that no steric hindrance is exerted upon the PMO when coupled to an α-helical peptide moiety.

[00118] More significantly the data demonstrates the fact that in the *in vivo* model of RNA splicing suppression the stabilized CPA enhanced cell entry into skeletal muscle.

[00119] Surprisingly, and very significantly, it has been demonstrated that tissues refractory to naked PMO transfection re-express dystrophin protein in both the heart and brain (purkinje cell) compartments when the PMO is conjugated with a StaP (Figures 12 and 13).

[00120] The repertoire of human and animal diseases that can be addressed is now expanded and enhanced due to the increased pharmacodynamics of the PMOs when conjugated with a stabilised peptide. Neuromuscular disease, metabolic disease, cancer, age-related degenerative diseases and acquired viral infection can all be targeted.

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CLAIMS

1. A drug carrying cell penetrating molecule (DCCPM) comprising:
    i. a biologically active compound (BAC), and
    ii. a cell penetrating agent (CPA), which BAC and CPA are linked directly or via a bi-functional linker (BFL),

    and wherein the CPA is a stabilized peptide (CPP) which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP), the StaP comprises a cross link or bridge between two conformationally adjacent amino acids of the peptide and the StiP comprises a cross link or bridge between at least three conformationally adjacent amino acids of the peptides and the BAC is an electrically neutral oligonucleotide (charge -1 to +1 at pH 7.5) (ON).

2. A DCCPM as claimed in claim 1 wherein the cross link or bridge comprises two components, a hydrocarbon bridge and a terminal methyl group.

3. A DCCPM as claimed in claim 1 wherein the peptide comprises at least two un-natural amino acids bearing all-hydrocarbon tethers (*e.g.* α-methyl,α-pentenyl glycine).

4. A DCCPM as claimed in claim 2 wherein CPA is stabilized with a staple or stitch incorporating one or more of: a (*S*)-pentenylalanine (**S5**) or its enantiomer (**R5**), a *S*-octenylalanine (**S8**) or its enantiomer (**R8**) or combinations thereof (e.g *R*-octenylalanine/*S*-pentenylalanine (**R8**/**S5**) or *S*-octenylalanine/*R*-pentenylalanine (**S8**/**R5**).

5. A DCCPM as claimed in claim 1 comprising a cross link or bridge between one or more of the unnatural amino acids of Table 1.

6. A DCCPM as claimed in claim 5 wherein the stabilized peptide comprises two or more olefin bearing side chains that are covalently formed.

7. A DCCPM as claimed in claim 1 wherein the stabilized conformation comprises at least one alpha helix.

8. A DCCPM as claimed in claim 1 wherein the stabilized conformation comprises at least one beta sheet.

9. A DCCPM as claimed in claim 1 wherein the stabilized conformation comprises at least one alpha helix and one beta sheet.

10. A DCCPM as claimed in claim 1 wherein the ON is an anti-sense oligonucleotide (AON).

11. A DCCPM as claimed in claim 10 wherein the ON is an anti-sense oligonucleotide is a polynucleic acid (PNA).

12. A DCCPM as claimed in any of claims 1 to 10 wherein the ON is a phosphorodiamate morpholino oligonucleotide (PMO).

13. A DCCPM as claimed in any of the preceding claims wherein the BAC alters the expression of an endogenous or exogenous gene.

14. A DCCPM as claimed in claim 13 wherein the endogenous gene targets a neuromuscular disease, a metabolic disease, cancer, an age-related degenerative disease or an acquired viral infection.

15. A DCCPM as claimed in any of the preceding claims wherein the BFL comprises a chemistry selected from the chemistries of Table 4.

16. A DCCPM as claimed in claim 15 comprising an amine to sulphydryl cross linker containing N- hydroxysuccinimide esters and malemide reactive groups separated by a cyclohexane spacer.

17. A DCCPM as claimed in claim 15 or 16 wherein the BFL is a succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC).

18. A DCCPM as claimed in claim 15 wherein the CPA is linked to a first end of the BFL covalently.

19. A DCCPM as claimed in claim 15 wherein the BAC is linked to a second end of the BFL covalently or non-covalently.

20. A method for facilitating the uptake of a biologically active compound (BAC), which is is an electrically neutral oligonucleotide (charge -1 to +1 at pH 7.5) (ON), into a cell by the conjugation of the oligonucleotide (ON) to a cell penetrating agent (CPA) which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP), the StaP

comprises a cross link or bridge between two conformationally adjacent amino acids of the peptide and the StiP comprises a cross link or bridge between at least three conformationally adjacent amino acids of the peptides directly or via a bi-functional linker (BFL) to form a drug carrying cell penetrating molecule (DCCPM) and presenting said DCCPM to said cell in a suitable vehicle.

21. A DCCPM as claimed in any of claims 1-15 for use in the treatment of a disease requiring alteration of the expression of an endogenous or exogenous gene.

22. A DCCPM as claimed in claim 21 for use in the treatment of neuromuscular disease, a metabolic disease, cancer, an age-related degenerative disease or an acquired viral infection.

23. A DCCPM as claimed in claim 21 for use in the treatment of Duchenne's muscular dystrophy.

24. A DCCPM as claimed in claim 23 wherein the DCCPM comprises an AON targeting exon 51 of the dystrophin gene.

25. A method of improving the bioavailability of a drug or biologically active compound (BAC), which is is an electrically neutral (charge -1 to +1 at pH 7.5) oligonucleotide (ON), comprising linking the ON to a stabilized peptide (CPP) which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP), the StaP comprises a cross link or bridge between two conformationally adjacent amino acids of the peptide and the StiP comprises a cross link or bridge between at least three conformationally adjacent amino acids of the peptides. .

26. A composition comprising a DCCPM as claimed in any of claims 1-24 and one or more pharmaceutically acceptable excipients.

27. A composition as claimed in claim 26 which is for administration orally, parenterally, intravenously or topically.