# IMPROVEMENTS IN DRUG DELIVERY

**[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), more preferably still electrically low charge carrying oligonucleotides (charge -3 to +3 at pH 7.5) and most preferably electrically neutral oligonucleotides (charge -1 to +1 at pH 7.5), such as, but not limited to, polynucleic acids (PNAs), phosphorodiamidate morpholino oligonucleotides (PMO's) or modified derivatives thereof.

**[0005]** The preferred BFL may be PEGylated, comprising poly ethylene glycol (PEG) groups including modifications such as an amine group, or incorporate a spacer, such as β-Ala. These modifications can improve solubilisation or provide appropriate spacing between functional moieties.

**[0006]** 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.

**[0007]** Still further aspects will be apparent from the detailed description.

## BACKGROUND TO THE INVENTION

**[0008]** 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.

**[0009]** An example of drugs or BACs which are delivered in a targeted manner are oligonucleotides (ON's), which term includes ON analogues.

**[0010]** ON's can target essential DNA, RNA and protein 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.

**[0011]** 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.

**[0012]** 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].

**[0013]** 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 or other cellular processes (*e.g.* post-translational modifications).

**[0014]** An advantage of steric blocking based suppression over that of siRNA/RNAi based RNase H-induction of the RNA Induced Silencing Complex is the reduced likelihood of off target side effects.

**[0015]** Modifications of an ON to produce a negatively charged backbone improve stability[4-7], but these backbone chemistries *e.g.* 2'O-Methyl Phosphothioate analogues, elicit membrane toxicity issues, cause thrombocytopaenia and injection site problems upon clinical translation[8], such that efficacy is prevented by toxicity issues, even when administration protocols become increasingly intermittent[9].

**[0016]** Indeed WO2013/150338 and WO 2014/053622 both disclose delivering negatively charged ON's of small size (typically smaller than 1.5KDa) by complexing them with positively charged linear or stapled peptides of equal or greater than 15 amino acids and in the range of 15-27 amino acids.

**[0017]** JACS, Vol 136, 2014, GJ Hilnski et al, describe stapled and stitched peptides that are able to penetrate cells. Reference is made to the possibility that these peptides could be used to deliver an oligonucleotide, presumably in the same manner as disclosed in the international applications disclosed above, i.e. by complexation. There is nothing to suggest creating new entities of much larger size (greater than 1.5KDa, through 2.5KDa, 5KDa, 7.5KDa, 10KDa, 12.5KDa or more) by covalently linking a BAC with a CPA, optionally via a BFL and indeed, the prior methodology requires the respective components to have opposite charges to facilitate complexing.

**[0018]** The use of electrically low charge carrying oligonucleotides (charge -3 to +3 at pH 7.5) and most preferably electrically neutral oligonucleotides (charge -1 to +1 at pH 7.5), such as, but not limited to, polynucleic acids (PNAs), phosphorodiamidate morpholino oligonucleotides (PMO's), (covalently) conjugated directly or indirectly, using a BFL, was not apparent and indeed, limiting the charge on the ON further allows the use of smaller peptides (less than 15 amino acids in length, through 14, 13, 12, 11, 10, 9, 8, 7, 6 to as few as 4 or 5) as carriers.

**[0019]** The use of uncharged ON backbones, such as phosphorodiamidate morpholino oligonucleotides (PMOs), represent attractive BAC's as they have an impeccable safety record in a preclinical and clinical setting.[2,10-13]

**[0020]** However, their ability to penetrate cells and access their targets is compromised due to their uncharged nature[14].

**[0021]** Overcoming the problem of facilitating their entry into cells is therefore desirable.

**[0022]** 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.

**[0023]** The approach has generally been to use charged peptides as non-covalent complexes to facilitate cell entry of a BAC. Conjugation has also been tried.

**[0024]** W02014/064258 is an example of the existing conjugating art. A negatively charged ON is coupled to a targeting peptide via a linker. The targeting peptide is a receptor targeting moiety, and not a stapled or stitched peptide, and indeed considerable doubt exists as to whether DNA or RNA molecules can gain cell entry using a receptor targeting moiety as once a negatively charged ON is bound to such a moiety, non-covalent interactions alter its conformation[15].

**[0025]** WO89/03849 discloses oligonucleotide-polyamide conjugates. There is no disclosure of the use of stitched or stapled peptides. The methodology described uses oligonucleotides as a scaffold for the chain extension of peptides and not as a conjugate for delivery, per se.

**[0026]** WO2011/131693 describes nucleic acid constructs which contain a nucleic acid specific for a given target gene and a selective inhibitor of a neurotransmitter transporter. There is no disclosure of the use of stitched or stapled peptides as a delivery agent.

**[0027]** 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).

**[0028]** However, since their first description[16] 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/][17], 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[18] and in higher mammals this manifests as prohibitive toxic side effects, such as tubular degeneration of the kidney[19].

**[0029]** At a physiological pH, and based on pKa of amino acid R groups, a formal charge (FC) can be calculated based on the formula:

$$FC = V - N - \frac{B}{2}$$

Where, V = valence electrons of the neutral atom in isolation; N = the number of non-bonding valence electrons on the defined atom; B = the total number of electrons shared in bonds.

**[0030]** Indeed, based on this, the CPPs typically used to date harbour many positively charged residues. It has been shown that there is a correlation between this positive charge and membrane toxicity[20].

**[0031]** Therefore, CPPs with a lower amount of positively charged residues within the amino acid sequence, whilst retaining the ability to cross a biological membrane, will be more clinically relevant.

**[0032]** 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 for CPA's[21,22].

**[0033]** They have illustrated this by delivering an ON targeted to repair a gene producing dystrophin. Targeting specific genes with ON is of course in itself known, as illustrated by, for example, WO2009/054725 and WO2010/123369. These publications however use a negatively charged backbone and deliver the cargo directly or using complexation.

**[0034]** 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 [denoted as StaP herein]) or two or more (stitched [denoted as StiP herein]) 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**).

**[0035]** 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 [21,23]. Given that StiP's and StaP's uptake is abrogated with reduced cellular decoration of heparin sulphate [21] a macropinocytotic entry mechanism is infered[24], suggesting this altered entry mechanism enables enhanced cellular uptake and bio-distribution compared to the state of the art.

**[0036]** Relative to their unmodified peptide precursors, 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[25]. 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 reduced or lack of membrane toxicity and immunogenicity enhances the clinical translatability of compounds when conjugated to drugs and BAC's such as ON's.

**[0037]** The BAC and CPP can be covalently conjugated directly, or covalently conjugated via a BFL. Many functional groups may be used for conjugation reactions.

**[0038]** 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.

**[0039]** 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 Arena viruses; 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.

**[0040]** Some tissues are particularly refractory to naked PMO transfection, *e.g.* heart, which may reflect differential vesicle-mediated PMO uptake mechanisms[23]. In fact, direct intra-cardiac injection of naked PMO does not even lead to efficient transfection[26], and refractory tissues tend to require repeat administration or high dose strategies[27-29]. However, whilst CPP conjugation

improves PMO bio-distribution and serum stability[30-32], toxicity is still a major roadblock for pipeline development[19].

**[0041]** 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.

**[0042]** Thus, providing DCCPM's which are able to deliver a drug or BAC more efficiently or to a target site, or with lower toxicity and immunogenicity would be highly desirable.

## BRIEF SUMMARY OF THE DISCLOSURE

**[0043]** 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).

**[0044]** A stapled peptide (StaP) may be formed by, for example, stapling two conformationally adjacent amino acids together, and a stitched peptide (StiP) may be formed by, for example, stitching at least three conformationally adjacent amino acids to form a stitched peptide (StiP).

**[0045]** The stapling or stitching results in the formation of a cross link or bridge between two conformationally adjacent amino acids of the peptide.

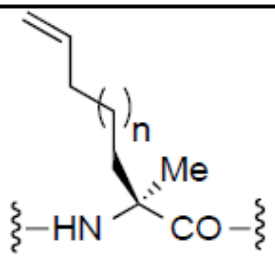
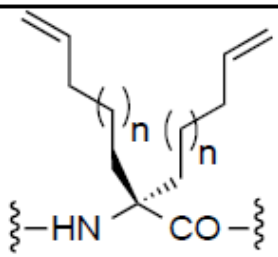
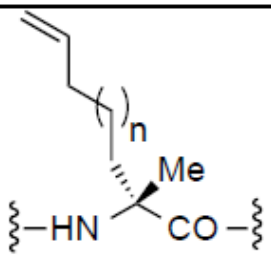
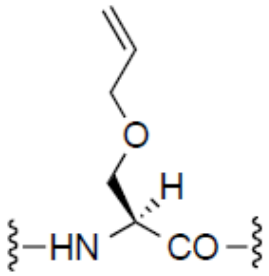
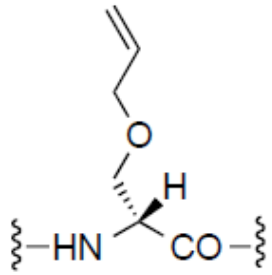
**[0046]** In a preferred embodiment the cross link or bridge comprises two components, a hydrocarbon bridge and a terminal methyl group. The hydrocarbon bridge may be composed of a double hydrocarbon bond or a single hydrocarbon bond.

**[0047]** The CPP preferably comprises at least two un-natural amino acids bearing all-hydrocarbon tethers (*e.g.* α-methyl,α-pentenyl glycine).

**[0048]** 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**).

**[0049]** 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.

**[0050] 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** | |

**[0051] Table 2**

| Entry | Peptide Sequence | Length | Peptide Type |
|---|---|---|---|
| 3 | TRQARRNRRRRWRRAAAA | 18 | Non RCM |
| 4 | TRQARRNRRRRWRERQR | 17 | Non RCM |
| 5 | RQIKIWFQNRRMKWKK | 16 | Non RCM |
| 6 | RRRRRRRRWRRR | 12 | Non RCM |
| 7 | LSQETFSDLWKLLPEN | 16 | Non RCM |
| 8 | NQLKRSFFALRDQI | 14 | Non RCM |
| 9 | NQLKRSFFALRDQI | 14 | Non RCM |
| 10 | TILKASVDYIRKLQREQQRAKEL | 23 | Non RCM |
| 11 | RRRRRRRRWRRR | 12 | Non RCM |
| 12 | RRRRRRRRRRRR | 12 | Non RCM |
| 13 | YGRKKRRQRRRP | 12 | Non RCM |
| 14 | RKFKRLFQ | 8 | Non RCM |
| 15 | NELKRSFFALRDQI | 14 | Non RCM |
| 16 | NQL-R8-RS-FFAL-S5-DQI | 14 | Non RCM |
| 17 | KNHTHQQDI | 9 | Non RCM |
| 18 | NELKRSFFALRDQIPSLQGEKASRAQILDKA TEYIQYNLRRK | 42 | Non RCM |
| 19 | KATEYIQYNLRRKNHTHQQDIDDL | 24 | Non RCM |
| 20 | ASTLFETFYLGGLLG | 15 | Non RCM |

| | | | |
|---|---|---|---|
| 21 | RRGSRPSGA-S5-RRR-S5-R | 15 | Non RCM |
| 22 | FNINDRIKELGTLI | 14 | Non RCM |
| 23 | DHIKDSFHSLRDSVPSLQGEKASRAQILDK ATEYIQYNLRRK | 42 | Non RCM |
| 24 | EYIQYNLRKNHTHQQDIDDLKRQNALLEQQ VRALGG | 36 | Non RCM |
| 25 | SSLFERFYNLVTPAGG | 16 | Non RCM |
| 26 | NSSFADFFHTVPYNLL | 16 | Non RCM |
| 27 | TRQARRN-S5-RRR-S5-RR | 14 | Non RCM |
| 28 | RRGSRPSGA-S5-RRR-S5-RAAAA | 19 | i,i+4 Staple |
| 29 | S5-RRQ-S5-RRDRQRRRRR | 15 | i,i+4 Staple |
| 30 | TRQ-S5-RRQ-S5-RRRWRERQR | 17 | i,i+4 Staple |
| 31 | SEELV-S5-EAH-S5-LCTLLENAIQDTVREQ | 26 | i,i+4 Staple |
| 32 | SEELVAEAH-S5-LCT-S5-LENAIQDTVREQ | 26 | i,i+4 Staple |
| 33 | SEELVAEAHNLCTLLE-S5-AIQ-S5-TVREQ | 26 | i,i+4 Staple |
| 34 | DRRQRRR-S5-RQR-S5-RRR | 15 | i,i+4 Staple |
| 35 | S5-RRQ-S5-RRRRQRRRRR | 15 | i,i+4 Staple |
| 36 | S-S5-ELV-S5-EAHNLCTLLENAIQDTVREQ | 26 | i,i+4 Staple |
| 37 | SEELVAEA-S5-NLC-S5-LLENAIQDTVREQ | 26 | i,i+4 Staple |
| 38 | SEELVAEAHNLC-S5-LLE-S5-AIQDTVREQ | 26 | i,i+4 Staple |
| 39 | SEELVAEAHNLCTLLENAI-S5-DTV-S5-EQ | 26 | i,i+4 Staple |
| 40 | FS-S5-LWK-S5-L | 8 | i,i+4 Staple |
| 41 | FM-S5-YWK-S5-L | 8 | i,i+4 Staple |
| 42 | QTFS-S5-LWK-S5-L | 10 | i,i+4 Staple |
| 43 | PPKKFR-S5-LFF-S5-S | 12 | i,i+4 Staple |
| 44 | KK-pff-R-S5-LFF-S5-S | 10 | i,i+4 Staple |
| 45 | RK-pff-S5-RLF-S5-SY | 10 | i,i+4 Staple |
| 46 | RKF-S5-RLF-S5-SY | 10 | i,i+4 Staple |
| 47 | R-pff-K-S5-RLF-S5-SY | 10 | i,i+4 Staple |
| 48 | AM-S5-YWK-S5-L | 8 | i,i+4 Staple |
| 49 | QTFSD-R5-WK-S5-L | 10 | i,i+4 Staple |
| 50 | KKFR-S5-LFF-S5-S | 10 | i,i+4 Staple |
| 51 | RRLFR-S5-NLFL-S5-T | 12 | i,i+4 Staple |
| 52 | RR-pff-S5-RLF-S5-SY | 10 | i,i+4 Staple |
| 53 | RKA-S5-RLF-S5-SY | 10 | i,i+4 Staple |
| 54 | RK-pff-S5-RLF-S5-SY | 10 | i,i+4 Staple |
| 55 | S5-RLF-S5-SY | 7 | i,i+4 Staple |
| 56 | KQKRKFS-S5-FFK-S5-L | 13 | i,i+4 Staple |
| 57 | KQKRK-pff-S-S5-FFK-S5-L | 13 | i,i+4 Staple |
| 58 | KQKRK-pff-S-S5-FFK-S5-L | 13 | i,i+4 Staple |
| 59 | KF-S5-RLF-S5 | 7 | i,i+4 Staple |
| 60 | S5-RLF-S5 | 5 | i,i+4 Staple |
| 61 | RKF-S5-RLF-S5 | 8 | i,i+4 Staple |
| 62 | KQKRKFS-S5-FFK-S5-LV | 13 | i,i+4 Staple |
| 63 | KQ-pff-RKKS-S5-FFK-S5-L | 13 | i,i+4 Staple |
| 64 | RK-pff-S5-RLF-S5 | 8 | i,i+4 Staple |

| | | | |
|---|---|---|---|
| 65 | F-S5-RLF-S5 | 6 | i,i+4 Staple |
| 66 | KTYRGAFQ-S5-LFQ-S5-VRE | 16 | i,i+4 Staple |
| 67 | STALR-S5-LIE-S5-LVNITQNQKAPL | 22 | i,i+4 Staple |
| 68 | STALRELI-S5-ELV-S5-ITQNQKAPL | 22 | i,i+4 Staple |
| 69 | STALRELIEEL-S5-NIT-S5-NQKAPL | 22 | i,i+4 Staple |
| 70 | NELK-S5-SFF-S5-LRDQIPELENNEKAP | 24 | i,i+4 Staple |
| 71 | LENRQ-S5-KLE-S5-ANRHLL | 16 | i,i+4 Staple |
| 72 | IL-S5-ASV-S5-YIRKLQREQ | 16 | i,i+4 Staple |
| 73 | FNI-S5-DRI-S5-ELGTLI | 14 | i,i+4 Staple |
| 74 | KN-S5-THQ-S5-DI | 9 | i,i+4 Staple |
| 75 | STALRELIEE LV-S5-ITQ-S5-QKAPL | 21 | i,i+4 Staple |
| 76 | NELK-S5-SFF-S5-LRDQI | 14 | i,i+4 Staple |
| 77 | LENRQKKLE-S5-ANR-S5-LL | 16 | i,i+4 Staple |
| 78 | ILKAS-S5-DYI-S5-KLQREQ | 16 | i,i+4 Staple |
| 79 | DHIK-S5-SFH-S5-LRDSV | 14 | i,i+4 Staple |
| 80 | DHIKDSF-S5-SLR-S5-SV | 14 | i,i+4 Staple |
| 81 | S5-YIQ-S5-NLRRKNHTHQQDIDDLLKRQNALLEQQVRALGG | 38 | i,i+4 Staple |
| 82 | TYRGAAQ-S5-AAQ-S5-VREV | 16 | i,i+4 Staple |
| 83 | TY-S5-GAF-S5-NLFQSVREV | 16 | i,i+4 Staple |
| 84 | A-S5-SVF-S5-NYFHSVPYFEL | 17 | i,i+4 Staple |
| 85 | GAF-S5-NLF-S5-SV | 10 | i,i+4 Staple |
| 86 | S5-GAF-S5-NLF-R5-SV | 11 | i,i+4 Staple |
| 87 | SYRGAFQ-S5-LFQ-S5-VREV | 16 | i,i+4 Staple |
| 88 | SSVFY-S5-YFH-S5-VPYFEL | 16 | i,i+4 Staple |
| 89 | A-S5-TLF-S5-TFYLGGLLG | 15 | i,i+4 Staple |
| 90 | S5-GAF-S5-NLFQSV | 11 | i,i+4 Staple |
| 91 | A-S5-SSF-S5-DFFHTVPYNLL | 17 | i,i+4 Staple |
| 92 | ERLRRRI-S5-LCR-S5-HHST | 16 | i,i+4 Staple |
| 93 | ERLRRRI-S5-NLCR-S5-HHST | 17 | i,i+4 Staple |
| 94 | ERLRRRL-S5-LCR-S5-HHST | 16 | i,i+4 Staple |
| 95 | ERLRRRF-S5-LCR-S5-HHST | 16 | i,i+4 Staple |
| 96 | ERFRRRI-S5-LCR-S5-HHST | 16 | i,i+4 Staple |
| 97 | ERLARRI-S5-LCR-S5-HHST | 16 | i,i+4 Staple |
| 98 | ENPESILD-S5-HVQ-S5-VM | 15 | i,i+4 Staple |
| 99 | PE-S5-ILD-S5-HVQRVM | 13 | i,i+4 Staple |
| 100 | ERLRRRI-S5-FCR-S5-HHST | 16 | i,i+4 Staple |
| 101 | ERLRRRNL-S5-LCR-S5-HHST | 17 | i,i+4 Staple |
| 102 | ERNLRRRI-S5-LCR-S5-HHST | 17 | i,i+4 Staple |
| 103 | ERWRRRI-S5-LCR-S5-HHST | 16 | i,i+4 Staple |
| 104 | RELRREI-S5-LCR-S5-HHST | 16 | i,i+4 Staple |
| 105 | ENPE-S5-ILD-S5-HVQRVM | 15 | i,i+4 Staple |
| 106 | NPE-S5-ILD-S5-HVQRVM | 14 | i,i+4 Staple |
| 107 | WPE-S5-ILD-S5-HVQRVM | 14 | i,i+4 Staple |
| 108 | PE-S5-ILD-S5-HVRRVMR | 14 | i,i+4 Staple |

| | | | |
|---|---|---|---|
| 109 | RPE-S5-ILD-S5-HVRRVMR | 15 | i,i+4 Staple |
| 110 | TRQA-R8-RNRRRR-S5-RR | 14 | i,i+7 Staple |
| 111 | RRGSRPSGA-R8-RRRRRA-S5 | 17 | i,i+7 Staple |
| 112 | RRGSRPSGA-R8-RRRRRA-S5-AA | 19 | i,i+7 Staple |
| 113 | TRQARRN-R8-RRRWRE-S5-QR | 17 | i,i+7 Staple |
| 114 | RRRR-R5-RRRWRR-S8 | 12 | i,i+7 Staple |
| 115 | KPE-S5-ILD-S5-HVQRVM | 14 | i,i+7 Staple |
| 116 | WPE-S5-ILD-S5-HVRRVMR | 15 | i,i+7 Staple |
| 117 | RRRR-R8-RQRRRR-S5-RR | 14 | i,i+7 Staple |
| 118 | RRGSRPSGA-R8-RRRRRR-S5 | 17 | i,i+7 Staple |
| 119 | R8-RRQRRR-S5-RQRRRRR | 15 | i,i+7 Staple |
| 120 | TRQARRN- R5-RRRWRE-S8-QR | 17 | i,i+7 Staple |
| 121 | RRRR-R5-RRRRRR-S8 | 12 | i,i+7 Staple |
| 122 | YGRK-R5-RRQRRR-S8 | 12 | i,i+7 Staple |
| 123 | S-R8-ELVAEA-S5-NLCTLLENAIQDTVREQ | 25 | i,i+7 Staple |
| 124 | SEELVAEAH-R8-LCTLLE-S5-AIQDTVREQ | 26 | i,i+7 Staple |
| 125 | SEELVAEAHNLCT-R8-LENAIQ-S5-TVREQ | 26 | i,i+7 Staple |
| 126 | RQIKIW-R5-QNRRMK-S8-KK | 16 | i,i+7 Staple |
| 127 | RRRR-R5- RRRWRR-S8 | 12 | i,i+7 Staple |
| 128 | S-R8-ELVAEA-S5-NLCTLLENAIQDTVREQ | 26 | i,i+7 Staple |
| 129 | SE-R8-LVAEAH-S5-LCTLLENAIQDTVREQ | 26 | i,i+7 Staple |
| 130 | SEELVAEAHNLC-R8-LLENAI-S5-DTVREQ | 26 | i,i+7 Staple |
| 131 | SEELVAEAHNLCTLLE-R8-AIQDT V-S5-EQ | 26 | i,i+7 Staple |
| 132 | LSQETF-R8-DLWKLL-S5-EN | 16 | i,i+7 Staple |
| 133 | ILR-R8-AVSHMK-S5-LRGT | 15 | i,i+7 Staple |
| 134 | ILR-R8-AVSHMK-S5-LRGT | 15 | i,i+7 Staple |
| 135 | NEL-R8-RS FRSL-S5-DSI | 14 | i,i+7 Staple |
| 136 | NEL-R8-RS FRAL-S5-DQI | 14 | i,i+7 Staple |
| 137 | NEL-R8-RS FFAL-S5-DSI | 14 | i,i+7 Staple |
| 138 | NEL-R8-RS FFAL-S5-DQI | 14 | i,i+7 Staple |
| 139 | IL-R8-MA-VSHM-S5-SLRGT | 15 | i,i+7 Staple |
| 140 | NEL-R8-RS FRAL-S5-DSI | 14 | i,i+7 Staple |
| 141 | NEL-R8-RS FFSL-S5-DQI | 14 | i,i+7 Staple |
| 142 | WNEL-R8-RSFRSL-S5-DQI | 15 | i,i+7 Staple |
| 143 | NQR-R8-LSFFAL-S5-DQI | 14 | i,i+7 Staple |
| 144 | NQL-R8- RSFFAL-S5-DQI | 14 | i,i+7 Staple |
| 145 | NQR-R8- LSFFAL-S5-DQI | 14 | i,i+7 Staple |
| 146 | NQL-R8- LSFFAR-S5-DQI | 14 | i,i+7 Staple |
| 147 | NKL-R8-RS FFAL-S5-DQI | 14 | i,i+7 Staple |
| 148 | NEL-R8-RS FFAL-S5-DQI | 14 | i,i+7 Staple |
| 149 | NELK-R8-SFFALR-S5-QIPELENNEKAP | 24 | i,i+7 Staple |
| 150 | AHL-R8- LCLEKL-S5-GLV | 14 | i,i+7 Staple |
| 151 | NQL-R8-RSFFAL-S5-DQI (D-amino acids) | 14 | i,i+7 Staple |
| 152 | IQD-S5-LAFFSR-R8-LQN (D-amino acids) | 14 | i,i+7 Staple |
| 153 | NKL-R8-RS-FKAL-S5-KQI | 14 | i,i+7 Staple |

| | | | |
|---|---|---|---|
| 154 | NELK-R8-S-FFALR-S5-QI | 14 | i,i+7 Staple |
| 155 | NQL-R8-RS-FFAL-S5-DQIPELENNEKAP | 24 | i,i+7 Staple |
| 156 | NQL-R8-RSFFAL-S5-DQI | 14 | i,i+7 Staple |
| 157 | AHL-R8-LCLEKL-S5-GLV-(K-(PEG)1- | 15 | i,i+7 Staple |
| 158 | KV-R8-ILK KAT-S5-YILS | 14 | i,i+7 Staple |
| 159 | R8-KR RAHA-S5-AERARR | 14 | i,i+7 Staple |
| 160 | IQD-S5-LAFFSR-R8-LQN (D-amino acids) | 14 | i,i+7 Staple |
| 161 | NQL-R8-RSFFAL-S5-DQI (D-amino acids) | 14 | i,i+7 Staple |
| 162 | NQL-R8-RS-FFAL-S5-DQI | 14 | i,i+7 Staple |
| 163 | NQL-R8-RSFFAL-S5-DQI | 14 | i,i+7 Staple |
| 164 | EENAKRR-R8-HNALER-S5-RR | 17 | i,i+7 Staple |
| 165 | NQL-R8-FSRFAL-S5-DQI(D-amino acids) | 14 | i,i+7 Staple |
| 166 | NQL-R8-LS-S5-DQI | 10 | i,i+7 Staple |
| 167 | NQL-R8-FS-S5-DQI | 10 | i,i+7 Staple |
| 168 | TILKASVDYI RKL-R8-REQQRA-S5-EL | 23 | i,i+7 Staple |
| 169 | FNI-R8-DRI-S5-TLI | 11 | i,i+7 Staple |
| 170 | RNI-R8-DRI -S5-TRI | 11 | i,i+7 Staple |
| 171 | KATEYIQYNLRRKN-R8-THQQDI-S5-DL | 24 | i,i+7 Staple |
| 172 | NEL-R8-RSFFAL-S5-DQIDQIPAAKRVKLD | 26 | i,i+7 Staple |
| 173 | NQL-R8-RSFFAL-S5-DQI | 14 | i,i+7 Staple |
| 174 | RNI-R8-DRIKEL-S5-TLI | 14 | i,i+7 Staple |
| 175 | FNIN-R8-RIKELG-S5-LI | 14 | i,i+7 Staple |
| 176 | FNI-R8-DRIKEL-S5-TRI | 14 | i,i+7 Staple |
| 177 | NQL-R8-RS FRAL-S5-DQI | 15 | i,i+7 Staple |
| 178 | NEL-R8-RSFFAL-S5-DQIDQIPKKKRKV | 24 | i,i+7 Staple |
| 179 | ENPE-R8-ILDEHV-S5-RVM | 15 | i,i+7 Staple |
| 180 | S8-RQARRN-B5-RRRWRE-S8-QR | 16 | i,i+4, i+11 Stitch Reduced |
| 181 | TRQ-S5-RRN-B5-RRRWRE-S8-QR | 17 | i,i+4, i+11 Stitch |
| 182 | TRQ-S5-RRA-B5-RRRWRE-S8-QR | 17 | i,i+4, i+11 Stitch |
| 183 | S5-RRN-B5-RRRWRE-S8 | 12 | i,i+4, i+11 Stitch |
| 184 | EYIQ-R5-NLRRKNH-S8-HQQDIDDLKRQNALLEQQVRALGG | 37 | i,i+4, i+11 Stitch |
| 185 | S8-RQARRQ-B5-RRRWRE-S8-QR | 17 | i,i+4, i+11 Stitch Reduced |
| 186 | TRQ-S5-Q-B5-RRRWRE-S8-QR | 15 | i,i+4, i+11 Stitch |
| 187 | TRQ-S5-RRN-B5-RRRWRE-S8-QR | 17 | i,i+4, i+11 Stitch Reduced |
| 188 | R8-RQARRN-B5-RRRWRE-S8-QR | 17 | i,i+4, i+11 Stitch Reduced |
| 189 | R8-RQARRQ-B5-RRRWRE-S8-QR | 17 | i,i+4, i+11 Stitch |
| 190 | S5-RRN-B5-RRRWRR-S8 | 12 | i,i+4, i+11 Stitch |
| 191 | RRA-B5-RRRWRR-S8 | 11 | i,i+4, i+11 Stitch |
| 192 | S5-RRR-B5-RRRRRR-S8 | 12 | i,i+4, i+11 Stitch |
| 193 | S5-KIW-B5-QNRRNLK-S8 | 13 | i,i+4, i+11 Stitch |
| 194 | S5-RRR-B5-RRRRRR-S8 | 12 | i,i+4, i+11 Stitch |
| 195 | S5-GRK-B5-RRQRRR-S8 | 12 | i,i+4, i+11 Stitch |
| 196 | S5-RRQ-B5-RRRWRR-S8 | 12 | i,i+4, i+11 Stitch |

| | | | |
|---|---|---|---|
| 197 | S5-RRR-B5-RRRWRR-S8 | 12 | i,i+4, i+11 Stitch |
| 198 | RQ-S5-KIW-B5-QNRRMK-S8-KK | 16 | i,i+4, i+11 Stitch |
| 199 | S5-KIW-B5-QNRRAK-S8 | 12 | i,i+4, i+11 Stitch |
| 200 | S5-RRR-B5-RRRWRR-S8 | 12 | i,i+4, i+11 Stitch |
| 201 | L-S5-ILQ-B5-AVQVIL-S8-LEQQVRER | 21 | i,i+4, i+11 Stitch |
| 202 | LLILQQAV-S5-VIL-B5-LEQQVR-S8-R | 21 | i,i+4, i+11 Stitch |
| 203 | S5-DFS-B5-YWK-R5-L | 10 | i,i+4, i+11 Stitch |
| 204 | LS-S5-ETF-B8-DLWKLL-S8-EN | 16 | i,i+4, i+11 Stitch |
| 205 | LSQ-S5-TFS-B8-LWKLLA-S8-N | 16 | i,i+4, i+11 Stitch |
| 206 | L-S5-ILQ- B5-AVQ-R5-ILGLEQQVRER | 21 | i,i+4, i+11 Stitch |
| 207 | LLILQQAV-S5-VIL-B5-LEQ-R5-VRER | 21 | i,i+4, i+11 Stitch |
| 208 | LLIL-S5-QAV-B5-VIL-R5-LEQQVRER | 21 | i,i+4, i+11 Stitch |
| 209 | R5-DFS-B5-YWK-S5-L | 10 | i,i+4, i+11 Stitch |
| 210 | LS-S5-ETA-B8-DLWKLL-S8-EN | 16 | i,i+4, i+11 Stitch |
| 211 | EDIIRNIA- S5-HLA-B5-VGDWNLD-S8-SI | 23 | i,i+4, i+11 Stitch |
| 212 | NIA-S5-HLA-B5-VGDWNLD- S8-SI (isomer 2) | 18 | i,i+4, i+11 Stitch |
| 213 | S5-HLA-B5-VGDWNLD-S8 (isomer 1) | 13 | i,i+4, i+11 Stitch |
| 214 | NVKRR-R8-HNVLER-S5-RRNEL-R8-RSFFAL-S5-DQI | 29 | i,i+4, i+11 Stitch |
| 215 | S5-YIQ-B5-NLRRKNH-S8-HQQDIDDLLKRQNALLEQQVRALGG | 38 | i,i+4, i+11 Stitch |
| 216 | NIA-S5-HLA-B5-VGDWNLD-S8-SI | 18 | i,i+4, i+11 Stitch |
| 217 | NIA-S5-HLA-B5-VGDWNLD-S8 | 16 | i,i+4, i+11 Stitch |
| 218 | S5-HLA-B5-VGDWNLD-S8 | 13 | i,i+4, i+11 Stitch |
| 219 | EYIQYNLR-S5-KNH-B5-HQQDID-S8-LKRQNALLEQQVRALGG | 37 | i,i+4, i+11 Stitch |

S5= a-methyl, a-alkenylglycine with 5 carbon chain

S8= a-methyl, a-alkenylglycine with 8 carbon chain

B5= a-methyl, a-alkenylglycine with two 5 carbon chain

**[0052]** Alternative CPPs and their method of manufacture are disclosed in Chu *et al*, 2014 and associated supplementary information, and are incorporated by reference[21].

**[0053]** The exemplified stabilized peptide comprises two or more olefin bearing side chains that are covalently formed, typically by means of a ring-closing metathesis.

**[0054]** The stabilized conformation typically comprises at least one alpha helix. It may however, in the alternative, comprise at least one turn (for example, but not limited to, α, β, γ, δ or π), several turns to form a beta sheet, or a combination of one or more of: an alpha helix, turn, or beta sheet.

**[0055]** The formal charge of a CPP is calculated at physiological pH (about 7.5) and is based on the pKa of amino acid R groups. These values ($pK_x$) are represented in Table 3.

**[0056] Table 3**

| Name | 3-Letter Symbol | 1-Letter Symbol | Molecular weight | Molecular Formula | Residue Formula | Residue Weight (-$H_2O$) | $pK_a$ [1] | $pK_b$ [2] | $pK_x$ [3] | pI [4] |
|---|---|---|---|---|---|---|---|---|---|---|
| **Alanine** | Ala | A | 89.10 | $C_3H_7NO_2$ | $C_3H_5NO$ | 71.08 | 2.34 | 9.69 | — | 6.00 |
| **Arginine** | Arg | R | 174.20 | $C_6H_{14}N_4O_2$ | $C_6H_{12}N_4O$ | 156.19 | 2.17 | 9.04 | 12.48 | 10.76 |
| **Asparagine** | Asn | N | 132.12 | $C_4H_8N_2O_3$ | $C_4H_6N_2O_2$ | 114.11 | 2.02 | 8.80 | — | 5.41 |
| **Aspartic acid** | Asp | D | 133.11 | $C_4H_7NO_4$ | $C_4H_5NO_3$ | 115.09 | 1.88 | 9.60 | 3.65 | 2.77 |
| **Cysteine** | Cys | C | 121.16 | $C_3H_7NO_2S$ | $C_3H_5NOS$ | 103.15 | 1.96 | 10.28 | 8.18 | 5.07 |
| **Glutamic acid** | Glu | E | 147.13 | $C_5H_9NO_4$ | $C_5H_7NO_3$ | 129.12 | 2.19 | 9.67 | 4.25 | 3.22 |
| **Glutamine** | Gln | Q | 146.15 | $C_5H_{10}N_2O_3$ | $C_5H_8N_2O_2$ | 128.13 | 2.17 | 9.13 | — | 5.65 |
| **Glycine** | Gly | G | 75.07 | $C_2H_5NO_2$ | $C_2H_3NO$ | 57.05 | 2.34 | 9.60 | — | 5.97 |
| **Histidine** | His | H | 155.16 | $C_6H_9N_3O_2$ | $C_6H_7N_3O$ | 137.14 | 1.82 | 9.17 | 6.00 | 7.59 |
| **Hydroxyproline** | Hyp | O | 131.13 | $C_5H_9NO_3$ | $C_5H_7NO_2$ | 113.11 | 1.82 | 9.65 | — | — |
| **Isoleucine** | Ile | I | 131.18 | $C_6H_{13}NO_2$ | $C_6H_{11}NO$ | 113.16 | 2.36 | 9.60 | — | 6.02 |
| **Leucine** | Leu | L | 131.18 | $C_6H_{13}NO_2$ | $C_6H_{11}NO$ | 113.16 | 2.36 | 9.60 | — | 5.98 |
| **Lysine** | Lys | K | 146.19 | $C_6H_{14}N_2O_2$ | $C_6H_{12}N_2O$ | 128.18 | 2.18 | 8.95 | 10.53 | 9.74 |
| **Methionine** | Met | M | 149.21 | $C_5H_{11}NO_2S$ | $C_5H_9NOS$ | 131.20 | 2.28 | 9.21 | — | 5.74 |
| **Phenylalanine** | Phe | F | 165.19 | $C_9H_{11}NO_2$ | $C_9H_9NO$ | 147.18 | 1.83 | 9.13 | — | 5.48 |
| **Proline** | Pro | P | 115.13 | $C_5H_9NO_2$ | $C_5H_7NO$ | 97.12 | 1.99 | 10.60 | — | 6.30 |
| **Pyroglutamatic** | Glp | U | 139.11 | $C_5H_7NO_3$ | $C_5H_5NO_2$ | 121.09 | — | — | — | 5.68 |
| **Serine** | Ser | S | 105.09 | $C_3H_7NO_3$ | $C_3H_5NO_2$ | 87.08 | 2.21 | 9.15 | — | 5.68 |
| **Threonine** | Thr | T | 119.12 | $C_4H_9NO_3$ | $C_4H_7NO_2$ | 101.11 | 2.09 | 9.10 | — | 5.60 |
| **Tryptophan** | Trp | W | 204.23 | $C_{11}H_{12}N_2O_2$ | $C_{11}H_{10}N_2O$ | 186.22 | 2.83 | 9.39 | — | 5.89 |
| **Tyrosine** | Tyr | Y | 181.19 | $C_9H_{11}NO_3$ | $C_9H_9NO_2$ | 163.18 | 2.20 | 9.11 | 10.07 | 5.66 |
| **Valine** | Val | V | 117.15 | $C_5H_{11}NO_2$ | $C_5H_9NO$ | 99.13 | 2.32 | 9.62 | — | 5.96 |

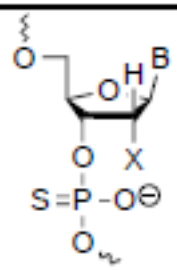
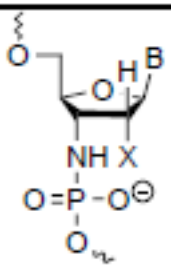
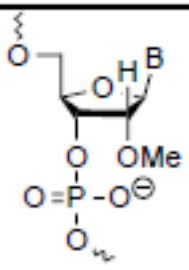
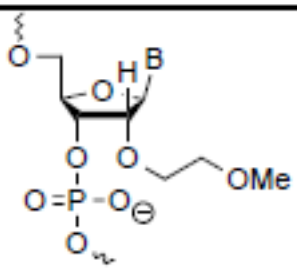
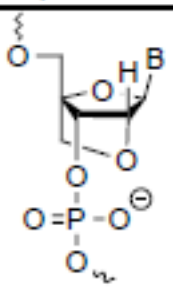
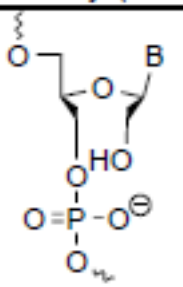
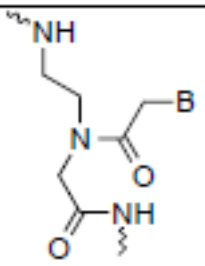
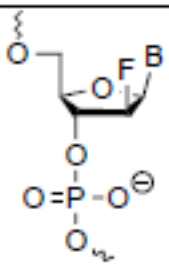
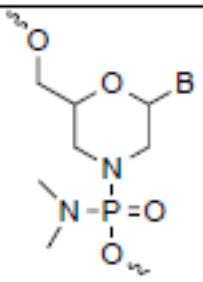
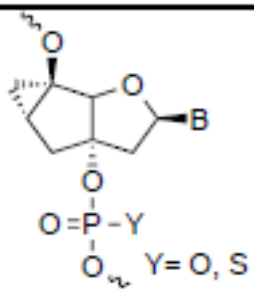
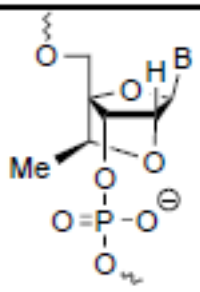
[1] $pK_a$ is the negative of the logarithm of the dissociation constant for the -COOH group
[2] $pK_b$ is the negative of the logarithm of the dissociation constant for the $-NH_3^+$ group
[3] $pK_x$ is the negative of the logarithm of the dissociation constant for any other group in the molecule
[4] pI is the pH at the isoelectric point
References: D. R. Lide, *Handbook of Chemistry and Physics, 72nd Edition*, CRC Press, Boca Raton, FL, 1991.

**[0057]** CPPs typically used to date harbour many positively charged residues. Reducing the amount of positively charged residues within the amino acid sequence, whilst retaining the ability to cross a biological membrane, will be more clinically relevant.

**[0058]** Accordingly, it is possible to reduce the charge on the peptide sequences illustrated in Table 2.

**[0059]** The preferred BAC is an oligonucleotide (ON), more preferably still an anti-sense oligonucleotide (AON). Different anti-sense oligonucleotide chemistries are illustrated in Table 4, with the use of low charge or neutral charged chemistries, such as, phosphorodiamidate morpholino oligonucleotides (PMOs) being preferred.

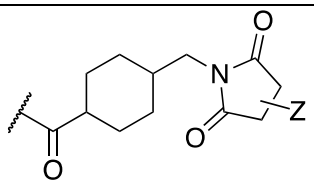
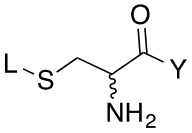
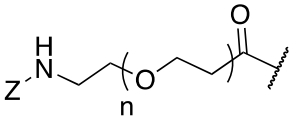
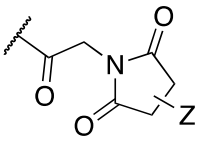
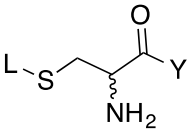
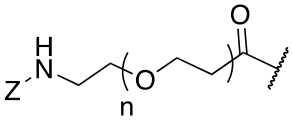
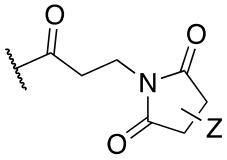
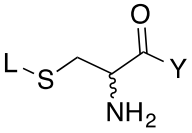
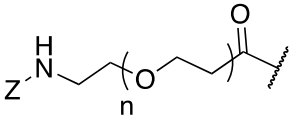
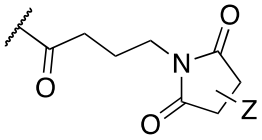
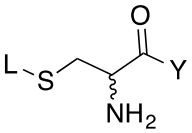
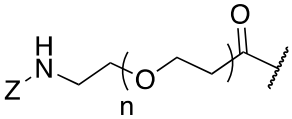
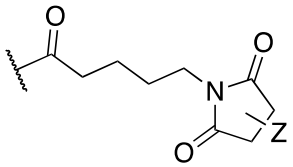
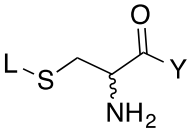
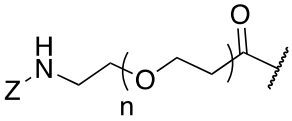
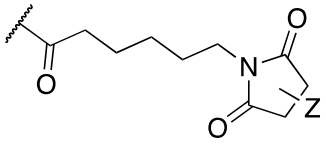
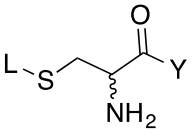
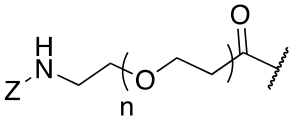
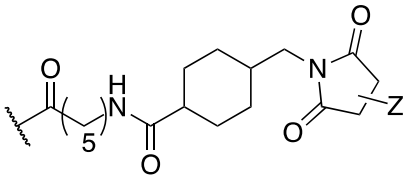
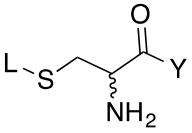
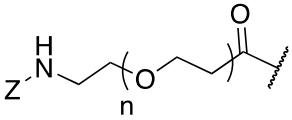
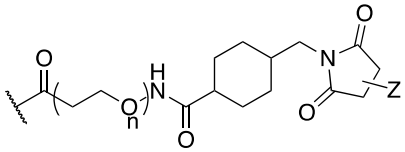
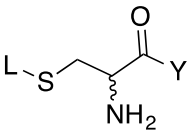
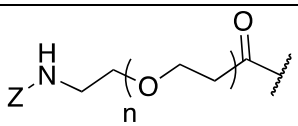
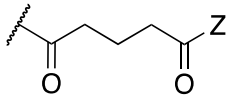
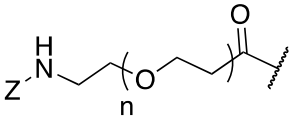
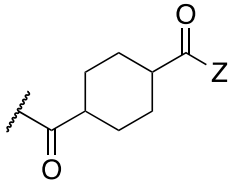
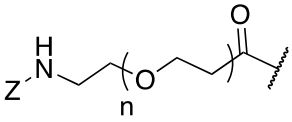
**[0060] Table 4**

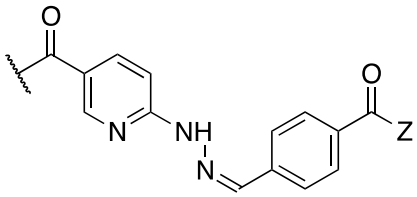
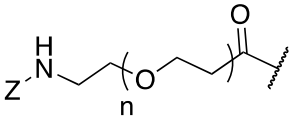
| | | |
|---|---|---|
|  Phosphorothioate (**PS**) |  N'3 Phosphoramidite (**NP**) |  2'-*O*-Methyl (**2' *O*-Me**) |
|  2'-*O*-Methoxyethyl (**MOE**) |  Locked Nucleic Acid (**LNA**) |  Unlocked Nucleic Acid (**UNA**) |
|  Peptide Nucleic Acid (**PNA**) |  2'F-Arabino Nucleic Acid (**2'F-ANA**) |  Phoshoramidate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

**[0061]** 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 disease *e.g.* viral infections.

**[0062]** Whilst the BAC may be linked to the CPP directly the Applicant has found the use of a BFL desirable. Exemplary, non-limiting BFL chemistries are illustrated in Table 5.

**[0063] Table 5**

| Entry | Linker (L) | Linker acronym is present | Z | $Y_3$ |
|---|---|---|---|---|
| 1 |  | SMCC |  |  or not present |
| 2 |  | AMAS |  |  or not present |
| 3 |  | BMPS |  |  or not present |
| 4 |  | GMPS |  |  or not present |
| 5 |  | DMVS |  |  or not present |
| 6 |  | EMCS |  |  or not present |
| 7 |  | LC-SMCC |  |  or not present |
| 8 |  | SM(PEG)$_n$ |  |  or not present |
| 9 |  | DSG | Not present |  or not present |
| 10 |  | DSCDS | Not present |  or not present |

| | | | | |
|---|---|---|---|---|
| 11 |  | HNA | Not present |  or not present |

**[0064]** By way of a footnote to Table 5, the following should be noted:

**[0065]** Figure 5A highlights general structure of a DCCPM where the following are preferred, but not limited to the following defined atoms or groups.

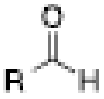
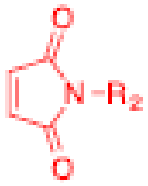
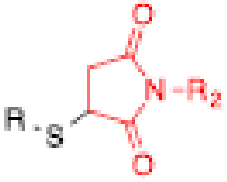
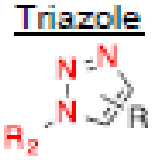
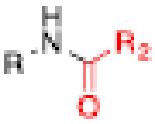
**[0066]** In a preferred embodiment illustrated in figure 5C, where Y1= Nitrogen, Y2 = Hydrogen, Y3 = spacer such as (PEG)n n=5, but not limited to those identified in Table 5, Z = a sulfur containing moiety e.g. Cysteine and L = BFL such as SMCC

**[0067]** Other embodiments may utilize variations over the structure shown in Figure 5A. For example if another embodiment does not require a thiol for conjugation of the BFL to the CPA as illustrated in Figure 5D, then Z = Y3 where Y3 is a spacer in Table 5. For a BFL that does not require a sulphur for conjugation of the BAC and CPA e.g. not limited to entries 9-11 in Table 5 Z= a covalent bond between L and Y3

**[0068]** Other embodiments may not require the use of a spacer, a BFL and as such a thiol group for the formation of a DCCPM depicted in Figure 7 then the following apply. If no spacer is utilized then Y3 can represent a covalent bond between Y1 and the BAC in which case Z and L = Y1 where Y1 is a N terminus of the CPA.

**[0069]** These chemistries may be further expanded and Table 6 exemplifies modifications to amino acids via which functional groups can be introduced to provide desirable properties to the DCCPM. These will include, but are not limited to, an acetyl, a cholesterol, a fatty acid, a polyethylene glycol, a polysaccharide, an aminoglycan, a glycolipid, a polyphenol, a nuclear localising signal, a nuclear export signal, an antibody, and a targeting molecule.

**[0070] Table 6.**

| Functional group (X) | Reacting Functional Group ($X^1$) | Resulting Functional Group |
|---|---|---|
| Aldehyde  | Hydrazine  | Hydrazone  |
| Thiol  | Maleimides  | Thioether  |
| Thiol  | Thiol  | Disulfide  |
| Alkyne  | Azide  | Triazole  |
| Amine  | Carboxylic acid  | Amide  |
| Diazirine  | Any | Various  |
| Olefin  | Olefin  | Olefin  or further reduction  |
| Aryl or Olefin R–M | Halide $R_2$–X | R–$R_2$ |

**[0071]** A preferred linker chemistry utilises an amine to sulphydryl cross linker containing N-hydroxysuccinimide esters and malemide reactive groups separated by a cyclohexane spacer namely succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) to form a covalent bond between the BFL and the CPP.

**[0072]** A schematic intermediate compound using SMCC as the BFL and the resultant DCCPM is depicted in Fig 5b.

**[0073]** In a particularly preferred embodiment the linker may incorporate polyethylene glycol in single or multiple units $(PEG)_n$, where n=1 to 10 PEG molecules.

**[0074]** Hereafter, where the CPP comprises the sequence RKF-S5-RLF-S5 and the BFL is a PEGylated SMCC, the resultant compound is termed CP8M.

**[0075]** Where the CPP comprises the sequence RKF-S5-RLF-S5 and the BFL is a PEGylated hydrazynal nicotinic acid (HNA), the resultant compound is termed HP8M.

**[0076]** Where the CPP comprises the sequence RKF-S5-RLF-S5 and the BFL is SMCC, the resultant compound is termed C8M.

**[0077]** Thus a CPA, such as Compound III (Fig 5b) may be covalently linked to a BFL, if required, preferentially incorporating $(PEG)_n$ where n=1-10.

**[0078]** Covalent linkage to the CPP may be via, for example, but not limited to, a β-ala or any other suitable moiety.

**[0079]** In the preferred embodiment, the $(PEG)_n$ is linked using a sulphur containing molecule *e.g.* cysteine, to enable covalent coupling as a PEGylated (SMCC). This in turn is covalently linked to a functional group on the BAC, in the preferred embodiment a primary amine, (Compound I), thus generating a DCCPM (Compound V).

**[0080]** According to a second aspect of the invention there is provided a method for facilitating the uptake of a biologically active compound (BAC) into a cell by the conjugation of the biologically active compound, directly or via a bi-functional linker (BFL), to a cell penetrating agent (CPA) which is a stabilized peptide which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP), to form a drug carrying cell penetrating molecule (DCCPM) and presenting said DCCPM to said cell in a suitable vehicle.

**[0081]** Where HNA has been incorporated into the terminal end of the CPP, to form a DCCPM in which the BAC is an ON, the ON has been modified to incorporate 4 formyl benzioic acid to facilitate covalent conjugation.

**[0082]** 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.

**[0083]** 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.

**[0084]** In one embodiment the DCCPM is used in the treatment of a muscular dystrophy e.g. Duchenne muscular dystrophy (DMD) although the skilled person will readily appreciate that the invention can be used to target a wide range of genes.

**[0085]** In the case of DMD the DCCPM may comprise an AON targeting exon 51 of the dystrophin gene.

**[0086]** In accordance with a fourth aspect of the present invention there is provided a method of improving the bioavailability of a drug or BAC comprising linking the drug or BAC to a CPP which is a stabilized peptide which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP).

**[0087]** 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 which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP) and administering it to a subject.

**[0088]** 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.

**[0089]** 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.

**[0090]** 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

**[0091]** Embodiments of the invention are further described hereinafter with reference to the accompanying Drawings, in which:

**[0092]** Fig 1a-c show a general schematic of a CPP which it has been stabilized by means of the incorporation of olefin-bearing α, α-di-substituted amino acids that permit a further chemical modification such that a cross link can be formed. Cross linking of two such non-natural amino acids is termed stapling; cross linking involving more than two non-natural amino acids is termed stitching. This schematic can be referenced against Tables 1 and 2 of this application.

**[0093]** Fig 1a is an example of a non-cyclised and an i,i+4 ring closing metathesis (RCM) using

Grubb's Gen 1 catalyst to form an StaP CPA;

**[0094]** Fig 1b is an example of a reduced RCM StaP using conventional reduction chemistry;

**[0095]** Fig 1c is a schematic showing a selection of different StaP or StiP RCM configurations and their corresponding starting positions;

**[0096]** Fig 2 exemplifies the structures of charge variants: 3+ (CP8M-3), 2+ (CP8M-2), 1+ (CP8M-1) and 0+ (CP8M-0) illustrating relative positions of charge on the StaP, however the positions and charge can be varied in any permutation or combination;

**[0097]** Fig 3 exemplifies the structure of an amino acid which can be incorporated into StaP or StiPs with varying functional groups as defined in Table 6. The functional groups can then be used for bio-conjugation;

**[0098]** Fig 4a is a representation of a RCM reaction to form a StaP;

**[0099]** Fig 4b shows the resulting CD spectrum of the StaP and non-cyclised stating peptide;

**[00100]** Fig 4c is a COSY NMR spectra of CP8M;

**[00101]** Fig 4d is a NOESY NMR Spectra of CP8M;

**[00102]** Fig 4e is a TOCSY NMR spectra of CP8M;

**[00103]** The Spectra were collected at room temperature in $H_2O$ spiked with 10% $D_2O$ and 10 mM sodium acetate;

**[00104]** Fig 5a is a schematic diagram of StaP DCCPM depicting variants of linkers and spacers as defined in Table 5;

**[00105]** Fig 5b is a schematic diagram of DCCPM depicting the synthetic steps for the conjugation of a StaP to a PMO. The PMO is modified to yield a 5' amine group (compound I); the heterobifunctional protein crosslinker Succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC; compound II) is attached to 5' amine group to yield compound IV; an *i*,*i*+4 stapled peptide (compound III) is conjugated to generate the final DCCPM (compound V);

**[00106]** Fig 5c is a DCCPM of CP8M conjugated to an ON using a SMCC linker (*e.g.* PMO-CP8M);

**[00107]** Fig 5d is a DCCPM of HP8M using a HNA linker (PMO-HP8M);

**[00108]** The FITC group here and elsewhere may be any other fluorescent label and is present merely to enable visualization;

**[00109]** Fig 6 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;

**[00110]** Fig 7 shows a general schematic diagram of a DCCPM in which a FITC labelled PMO is directly conjugated to a CPA through a shared nitrogen forming compound VII;

**[00111]** Figs 8a-o show the liquid chromatography-mass spectrometry conformation of the synthetic steps and the molecular masses of compounds depicted in Fig 1, Fig 2 and Fig 5. All PMOs in this application are fluorescently labelled unless specified otherwise.

**[00112]** Fig 8a is a LRMS(API-ES) mass spectra of CP8M-NC. Mass calculated for $C_{76}H_{130}N_{18}O_{16}S^{2+}$ $(M+2H)^{2+}$ 791.5 found 791.8;

**[00113]** Fig 8b is a LRMS(API-ES) mass spectra of CP8M. Mass calculated for $C_{74}H_{125}N_{18}O_{16}S^{+}$ $(M+H)^{+}$ 1554.9 found 1554.5;

**[00114]** Fig 8c is a LRMS(API-ES) mass spectra of C8M. Mass calculated for $C_{59}H_{95}N_{16}O_{10}S^{+}$ $(M+H)^{+}$ 1220.5 found 1220.1;

**[00115]** Fig 8d is a LRMS(API-ES) mass spectra of HP8M. Mass calculated for $C_{77}H_{125}N_{20}O_{16}^{+}$ $(M+H)^{+}$ 1586.9 found 1586.5;

**[00116]** Fig 8e is a HRMS(LQT-ESI) mass spectra of PMO. Mass calculated for $C_{332}H_{500}N_{153}O_{110}P_{25}$ (M) 9164.1675 found 9164.1882;

**[00117]** Fig 8f is a HRMS(LQT-ESI) mass spectra of PMO-SMCC. Mass calculated for $C_{344}H_{513}N_{154}O_{113}P_{25}$ (M) 9383.2570 found 9383.2704;

**[00118]** Fig 8g is a HRMS(LQT-ESI) mass spectra of PMO-CP8M. Mass calculated for $C_{418}H_{638}N_{172}O_{129}P_{25}S^{+}$ $(M+H)^{+}$ 10937.1806 found 10937.2377;

**[00119]** Fig 8h is a HRMS(LQT-ESI) mass spectra of NF-PMO-CP8M. Mass calculated for $C_{393}H_{619}N_{172}O_{121}P_{25}S$ (M) 10490.0372 found 10490.2268;

**[00120]** Fig 8i is a HRMS(LQT-ESI) mass spectra of PMO-HP8M. Mass calculated for $C_{417}H_{626}N_{173}O_{127}P_{25}$ (M) 10863.1285 found 10863.0716;

**[00121]** Fig 8j is a LRMS(API-ES) mass spectra of FITC 3+ (CP8M-3). Mass calculated $C_{92}H_{132}N_{18}O_{20}S^{2+}$ $(M+2H)^{2+}$ 920.5 found 921.0;

**[00122]** Fig 8k is a LRMS(API-ES) mass spectra of FITC 2+ (CP8M-2). Mass calculated $C_{92}H_{131}N_{17}O_{20}S^{2+}$ $(M+2H)^{2+}$ 913.0 found 913.6;

**[00123]** Fig 8l is a LRMS(API-ES) mass spectra of FITC 1+ (CP8M-1). Mass calculated $C_{92}H_{129}N_{14}O_{20}S^{+}$ $(M+H)^{+}$ 1783.1 found 1782.6;

**[00124]** Fig 8m is a LRMS(API-ES) mass spectra of FITC + (CP8M-0). Mass calculated $C_{92}H_{126}N_{11}O_{20}S^{-}$ $(M-H)^{-}$ 1738.1 found 1738.0;

**[00125]** Fig 8n is a HRMS(LQT-ESI) mass spectra of PMO-4-FB. Mass calculated for $C_{340}H_{503}N_{153}O_{112}P_{25}^{+}$ $(M-H)^{+}$ 9295.1802 found 9295.2168; and

**[00126]** Fig 8o is a HRMS(LQT-ESI) mass spectra of PMO-CP8M-NC. Mass calculated for $C_{420}H_{642}N_{172}O_{129}P_{25}S^{+}$ $(M-H)^{+}$ 10965.2112 found 10965.2162;

**[00127]** Figs 9a and 9b demonstrate the comparative conjugation efficiencies of SMCC and HNA linker systems;

**[00128]** Fig 9a shows the % Conversion of HP8M and PMO-4FB into PMO-HP8M analysed by UV spectroscopy. % Conversion was calculated by $A_{350}$ of the bis-aryl hydrozone bond based on the starting reaction concentration of 705 µM. UV profiles of corresponding starting materials (HP8M and PMO-4FB) and the resulting conjugate (PMO-HP8M); and

**[00129]** Fig 9b shows the % isolated yield of PMO-CP8M and PMO-HP8M.

**[00130]** Fig 10a shows florescence microscopy images demonstrating 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 a PEGylated SMCC; and the StaP was RKF-S5-RLF-S5. This configuration of DCCPM is depicted as PMO-CP8M in the subsequent figs; unconjugated PMO acts as a control. All compounds were added to U2OS cells maintained in culture, without transfection reagent for 4 hours;

**[00131]** Fig 10b is a graphical representation of the delivery of PMO and PMO-CP8M into a human osteosarcoma cell line (U2OS) maintained in culture without transfection reagent;

**[00132]** Fig 11 is the analysis of PMO uptake into HEK293T cells by flow cytometry. Cells were incubated without PMO or with 1 μM PMO, 1 μM PMO-C8M or 1 μM PMO-CP8M-NC (all fluorescein-labelled) at 37ºC for 4 hours. Fluorescence was measured after washing the cells with PBS.

**[00133]** Fig 12a shows flow cytometry analysis of HEK293T cells treated with FITC+3 (CP8M3), FITC+2 (CP8M-2) and FITC+1 (CP8M-1) without transfection reagent for 4 hours;

**[00134]** Fig 12b shows flow cytometry analysis of HEK293T cells treated with FITC+3 (CP8M3), FITC+2 (CP8M-2) and FITC+1 (CP8M-1) without transfection reagent for 4 hours. The graph represents mean fluorescent intensity of FL1;

**[00135]** Fig 13 shows fluorescence microscopy images either 5μM PMO-CP8M or 5μM PMO delivery into a mouse cell line that harbours the *mdx* mutation of the dystrophin gene (H2K *mdx*) maintained in culture, without transfection reagent, in which the PMO has a fluorescent label.

**[00136]** Fig 14 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 5μM PMO-CP8M but not those treated with 5μM PMO alone, in which the PMOs have a fluorescent label. 24 hours after incubation with PMO-CP8M or PMO, H2K *mdx* cells were recovered and RNA isolated. The RNA was reversed transcribed and an amplification between exon 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;

**[00137]** Fig 15 shows immuno-cytochemical staining for dystrophin from *Tibialis anterior* muscles of *mdx* mice following a single intramuscular injection of either 2.2 nmol PMO-CP8M or

2.2nmol PMO into the *Tibialis anterior* muscle (dose in respect of molarity of PMO). Muscles were recovered 7 day post administration;

**[00138]** Fig 16 shows a graphical representation of the number of skeletal muscle fibre being positive for an immuno-cytochemical staining for dystrophin from *Tibialis anterior* muscles of *mdx* mice injected with either 2.2 nmol PMO-CP8M or 2.2nmol PMO. All dose are given in respect of the molarity of PMO;

**[00139]** Figs 17 a-d show immuno-cytochemical staining for dystrophin from skeletal muscles of *mdx* mice:

**[00140]** Fig 17a is from the diaphragm following a single intraperitoneal injection of 1 μmol/kg PMO-CP8M or 1 μmol/kg PMO [* denotes non fluorescent labelled PMO]. Muscles were recovered 7 days post administration;

**[00141]** Fig 17b is following multiple intravenous injections - total 4.4 μmol/kg PMO-CP8M or 4.4 μmol/kg PMO;

**[00142]** Fig 17c is following multiple intraperitoneal injections - total 10.9 μmol/kg PMO-CP8M or 10.9 μmol/kg PMO; and

**[00143]** Fig 17d is a graphical representation of the number of skeletal muscle fibre being positive for an immuno-cytochemical staining for dystrophin following intraperitoneal administration. All dose are given in respect of the molarity of PMO [* denotes non fluorescent labelled PMO]. Muscles were recovered 14 days post administration;

**[00144]** Figs 18a-c show immuno-cytochemical staining for dystrophin from heart muscles;

**[00145]** Fig 18a is of mdx mice following a single intraperitoneal injection of 1 μmol/kg PMO-CP8M or 1 μmol/kg PMO. Muscles were recovered 7 days post administration;

**[00146]** Fig 18b is following multiple intraperitoneal injections - total 7.6 μmol/kg PMO-CP8M or 7.6 μmol/kg PMO. Muscles were recovered 14 days post administration;

**[00147]** Fig 18c is following multiple intraperitoneal injections - total 10.9 μmol/kg PMO-CP8M or 10.9 μmol/kg PMO;

**[00148]** Fig 18d is a graphical representation of the number of cardiac muscle fibre being positive for an immuno-cytochemical staining for dystrophin following a multiple intraperitoneal injections - total 7.6 μmol/kg PMO-CP8M or 7.6 μmol/kg PMO; and

**[00149]** Fig 18e is a graphical representation of the number of cardiac muscle fibre being positive for an immuno-cytochemical staining for dystrophin following multiple intraperitoneal injections - total 10.9 μmol/kg PMO-CP8M or 10.9 μmol/kg PMO. All dose are given in respect of the molarity of PMO [* denotes non fluorescent labelled PMO]. Hearts were recovered 14 days post administration;

**[00150]** Fig 19 shows immuno-cytochemical staining for dystrophin from heart muscles of

*mdx* mice following intraperitoneal injections of PMO-CP8M with a total cargo size of 8.7 KDa and PMO-CP8M with total cargo size of 9.2 KDa. Hearts were recovered 14 days post administration;

**[00151]** Fig 20 shows immuno-cytochemical staining for dystrophin from the cerebellum of *mdx* mice following a single intraperitoneal injection of 1 μmol/kg PMO-CP8M or 1 μmol/kg PMO. All dose are given in respect of the molarity of PMO [* denotes non fluorescent labelled PMO]. Cerebella were recovered 7 days post administration; and

**[00152]** Fig 21 demonstrates *in vivo* liver cell uptake of fluorescein-labelled PMO after intravenous administration of PMO or PMO-HP8M to mdx mice (single intravenous injection, 1 μmol/kg, analysed 2 weeks post-injection). Un-injected mdx mice were used as a negative control.

## DETAILED DESCRIPTION

**[00153]** The invention is illustrated with reference to a single example which proves the benefit of the claimed invention.

**[00154]** An exemplary drug carrying cell penetrating molecule (DCCPM) was produced with a FITC label in order to demonstrate cellular uptake (Example 1).

**[00155]** The exemplary DCCPM comprises:

i) a biologically active compound (BAC) – (see Table 4 for non-limiting examples);

ii) a cell penetrating agent (CPA) which is a stabilized peptide (See Table 2 for non-limiting examples); and

iii) a bi-functional linker (BFL) (see Table 5 for non-limiting examples).

**[00156]** The three components forming the DCCPM are described in more detail below, although as illustrated in Fig 5, the BAC and CPA can be linked directly (Fig 6).

### 1. **The Biologically Active Compound**.

**[00157]** 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 physiological pH – about 7.5) such as polynucleic acids (PNAs) or PMOs or their modified derivatives that might impart a small electric charge (either positive or negative).

**[00158]** 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.

**[00159]** 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.

**[00160]** Additionally, ON's can be designed to target 5' translation initiation start sites of endogenous or 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.

**[00161]** Also, ON can be designed to target 3' untranslated region of an endogenous transcript that alters the stability of the transcript. Such targets include, and are not limited to, poly adenylation and/or cleavage sites of the transcript.

**[00162]** 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.

**[00163]** Non-limiting exemplary ON chemistries are illustrated in Table 4.

**[00164]** In the non-limiting example illustrated, 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**

**[00165]** The cell penetrating agents of the invention are stabilized peptides.

**[00166]** The peptides may be stabilized by stapling, to form a stapled peptide (StaP), or by stitching to form a stitched peptide (StiP)

**[00167]** All-hydrocarbon staples and stitches may confer a property, *e.g.* an α-helical structure, protease resistance, cellular penetrance, and biological activity.

**[00168]** 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).

**[00169]** 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.

**[00170]** 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.

**[00171]** Preferred isomer/enantiomers will be enriched to give a greater proportion of one particular isomer or enantiomer. Embodiments thereof may be made of greater than 90%, 95%, 98% or 99%, by weight, of a preferred isomer/enantiomer.

**[00172]** Non-limiting examples of unnatural amino acids used in stabilising a peptide structure are illustrated in Table 1.

**[00173]** In one embodiment the applicant employs α,α-disubstituted unnatural amino acids bearing all-hydrocarbon tethers (*e.g.* α-methyl,α-pentenyl glycine).

**[00174]** 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.

**[00175]** 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 the specific peptides shown in Table 2, a person skilled in the art can easily envisage peptides with 3, 4, 5 or more turn stabilising staples.

**[00176]** The hydrocarbon bridge may be composed of a double hydrocarbon bond or a single hydrocarbon bond.

**[00177]** In one embodiment the cell penetrating agent has a stitch or staple peptide comprising the sequence RFK-S5-RLF-S5.

**[00178]** 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.

**[00179]** 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 compound.

**[00180]** Peptide sequence can include modified amino acids to include functional groups that permit the addition of other moieties. Non-limiting examples of such moieties include an acetyl, a cholesterol, a fatty acid, a polyethylene glycol, a polysaccharide, an aminoglycan, a glycolipid, a polyphenol, a nuclear localising signal, a nuclear export signal, an antibody and a targeting molecule.

**3. Bi-functional Linker**

**[00181]** A bi-functional linker may be used to link the BAC to the CPA.

**[00182]** Preferred linkers will link between, for example, an amine group on the BAC and a sulfhydryl (thiol) group (usually a cysteine residue) on the CPA terminus. 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 5.

**[00183]** Another preferred linker system is hydrazynal nicotinic acid (HNA), however if the BAC is a PMO, the PMO is modified to incorporate 4 formyl benzioic acid.

**[00184]** 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 5, entries 8 and 9).

**[00185]** Linkers may include other elements that confer a desirable property on the DCCPM *e.g.* spacer between ON and CPA or an element that will enhance solubility, for example a PEGylated element as illustrated in Figs 5c and 5d. Non-limiting examples are shown in Table 5.

**[00186]** The biologically active compound is 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 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**

**[00187]** 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[33]. 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[34,35].

**[00188]** 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[29,36-41], which led to clinical development programs[8,13].

**[00189]** Although intravenously administered PMOs have demonstrated a dose-dependent increase in dystrophin re-expression with some functional benefit[13,42], 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[27-29].

**[00190]** To date unmodified CPA conjugation improves PMO bio-distribution and serum stability[30-32], however toxicity is still a major roadblock for pipeline development[19].

**[00191]** 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**

Nuclear Magentic Resonance (NMR) Analysis of StaP

**[00192]** $^{1}$H NMR spectra was recorded using a Bruker Avance III 500 (500 MHz) spectrometer. Samples were dissolved in $H_2O$ with 10% $D_2O$ and 10 mM sodium acetate.

**[00193]** NOESY spectra were recorded with a 12626.263 Hz sweep width, 4096 complex points (DQD acquisition mode) in the direct dimension and 1024 indirect points (States-TPPI acquisition mode). A NOESY mixing time of 250 ms was used to provide cross peaks with high

signal to noise while largely avoiding spin diffusion. A pre-saturation pulse on water and a 3-9-19 pulse sequence with 20% Z-gradients (4,5) aided solvent suppression. TOCSY spectra were recorded with the same spectral width and resolution as the NOESY with a homonuclear Hartman-Hahn transfer using the MLEV17 sequence for an 80 ms mixing time (6). Two power levels were used for excitation (3 dB) and spinlock (12.2 dB). Water suppression was achieved as with the NOESY.

High Resolution Mass Spectroscopy

**[00194]** High-resolution mass spectra were recorded on a Thermo scientific LQT Orbitrap XL under electron spray ionization conditions (ESI) or where indicated under Atomospheric Pressure Ionisation (API) condition.

Circular Dichroism (CD) Spectroscopy

**[00195]** CD analysis was performed on an Applied Photophysics Chirascan Circular Dichroism spectrometer. Samples were dissolved in $D_2O$ at 0.125 W/W% and data acquired in triplicate at room temperature and subsequently averaged and smoothed using built in qCD software. Graphs were plotted by subtracting a blank $D_2O$ spectrum from the acquired data to provided blank correction.

Synthesis of PMO-CP8M and NF-PMO-CP8M

**[00196]** PMO (22.2 mg, 2.1 μM) was dissolved in PBS (400 μL, 1 x) and incubated at room temp after the addition of SMCC linker (6 mg, 18 μM, 9x excess) dissolved in MeCN 100 μL. After 45 mins the mixture was desalted using sephadex g25 hydrated in a PBS 1x and was also used as the eluent. RCM-C-PEG-8Mer (3 mg, 2.5 μM) was mixed immobilised TCEP (750 μL) for 1 h. The SMCC modified PMO was then desalted into PBS/MeCN (500 μL 4:1) and immediately the peptide was eluted from the immobilised TCEP and stirred at room temp for 3 hours before purification on a Waters HLB column.

**[00197]** The solution was loaded onto 4 HLB columns, and washed with milliQ water to remove any salts then 20% MeCN in water and finally PMO-CP8M was removed with 50% MeCN in water. The MeCN content was reduced by rotary evaporation and the conjugate subsequently freeze dried to yield the final lipholysed compound.

Synthesis of PMO-HP8M

Modification of PMO to PMO-4FB.

**[00198]** 4-FB (250 mg, 1.5 mM) was dissolved in DMF with COMU (1.2 g, 2.6 mM) and NHS (230 mg, 2.0 mM) and stirred for a few mins. Nb, 4-FB did not fully dissolve until DIEA was added. DIEA (0.54 mL 3.0 mM) was then added upon which the reaction mixture changed from colourless to pale yellow/orange. The reaction mixture was stirred for 1 h and monitored by TLC using 5% MeOH in DCM. The mixture was separated over DCM to remove DMF then purified by flash chromatography using DMC to elute the top spot staining positive with 2,4 DNP. Product was collected as an off white solid 112 mg (30 %).

**[00199]** PMO (30.4 mg, 3 μM) was added to a solution of 4-FB and dissolved in Carbonate buffer:MeCN (50% MeCN) and NHS activated 4-FB (10 mg, 32 μM) was added and stirred overnight. The mixture was then desalted using sephadex G25 superfine with water:MeCN as an eluent. MeCN was removed by rotary evaporation and the remaining eluent was then freeze dried. Freeze dried product yielded 24 mg 83% yield.

Conjugation of PMO-4FB to HP8M

**[00200]** HP8M was dissolved in milliq ultra pure water (100 μL) to give a solution of 12 mg/mL. Aldehyde modified PMO (7 mg, 0.76 μM) was dissolved in water/MeCN (300 μL, 1:1) and desalted using sephadex G25 superfine and water/MeCN (1:1) as the eluent. The collected fraction was then diluted to 1 mL total volume in water:MeCN mix (1:1) and PMO content was analysed by UV/vis and found to be 6.5 mg/mL or 705 μM. HNA peptide and Analine (10 mM final conc) was then added and UV/vis monitored for evidence of $A_{354}$ and used to calculate the conjugation of PMO to peptide.

PMO and Peptide Synthesis

**[00201]** 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. A non-ring closed peptide was also produced as a control.

Cell Culture and Transfection

**[00202]** U2OS cells (Human osteosarcoma) were cultured in high glucose DMEM supplemented with 10% foetal calf serum (Sigma, UK) at 37°C under an 8% CO2/92% air atmosphere.

**[00203]** $H_2K$ mdx mouse myoblasts were cultured at 33°C under a 8% $CO_2$/92% air atmosphere in high-glucose DMEM supplemented with 20% foetal 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 after 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.

**[00204]** HEK293T cells (Human embryonic kidney) were cultured in high glucose DMEM supplemented with 10% foetal calf serum (Sigma, UK) at 37°C under an 8% $CO_2$/92% air atmosphere.

**[00205]** U2OS cells were incubated with PMO or PMO-CP8M 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-CP8M at 5.0μM: HEK293T cells were incubated with CP8M (1.0μM, 10μM and 100μM) and PMO, PMO-CP8M-NC, PMO-C8M at 1.0μM. Levels of fluorescence was quantified at 494 nm to determine relative entry of respective compounds by microscopic or flow cytometry methodologies.

RNA Extraction and nested RT-PCR Analysis

**[00206]** 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

**[00207]** *mdx* mice, with access to chow and water *ad libitum,* were used in all experiments. 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-CP8M

**[00208]** Intramuscular administrations: *Tibialis anterior* muscles of mdx mice were injected with either 2.2nmol PMO or 2.2 nmol PMO-CP8M under isoflurane anaesthesia. Systemic administration: *mdx* mice were subject to a single or repeated intraperitoneal injections of PMO or PMO-CP8M (or non-fluorescently labelled variants) at doses ranging from 1 μmol/kg to 10.9 μmol/kg total delivery; alternatively, *mdx* mice were subject to single or repeated intravascular injections of PMO or PMO-CP8M ranging from 1 μmol/kg to 4.4 μmol/kg total. A series of tissues were recovered at the end of the experiment that included skeletal muscle, heart, brain and liver.

Histology and Immuno-cytochemistry

**[00209]** For skeletal muscle, heart and brain, 10μM cryosections were cut and dystrophin protein was detected using rabbit polyclonal antibody to dystrophin (ab15277; Abcam, Cambridge, UK). Routine haematoxylin and eosin staining was used to assess general pathology and morphology. For liver, 10μM cryosections were dried and embedded in fluorescence-compatible mounting medium (Dako), and general fluorescence was assessed microscopically at 494 nm.

Flow cytometry

**[00210]** Uptake of fluorescently-labelled PMO was determined by flow cytometry using an Accuri C6 flow cytometer. PMO-transfected cells were released with trypsin, washed in PBS and kept on ice before analysis. Cell fluorescence in single live cells was determined using FlowJo software after appropriate gating. Untreated cells were used to establish gating settings for the determination of the % fluorescein-positive cells.

Statistical Analysis

**[00211]** 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**

**[00212]** Circular dichroism and the nuclear magnetic resonance data confirmed that the ordered structure of the peptides was as expected and that the stapled peptides adopted an $\alpha$-helical structure (Fig 4).

**[00213]** The conjugation of PMO-SMCC with a CPP to form PMO-CP8M has consistently yielded an efficiency of 10%. Surprisingly, adopting a conjugation based upon a PMO modified to incorporate 4 formyl benzioic acid and hydrazynal nicotinic acid (HNA) incorporated into the terminal end of the CPP, increased the efficiency of conjugation to yield PMO-HP8M at 59% (Fig 9).

**[00214]** PMO was conjugated to the bi-functional linker (a PEGylated SMCC) and a CPP (RKF-S5-RLF-S5) as confirmed by mass spectrometry (Fig 7). Subsequently PMO and PMO-CP8M were transfected into a standard cell line (U2OS) to determine if the CP8M conferred enhanced cell entry to the cell. Naked PMO were refractory to cell entry, giving only a background fluorescence signal, compared to a dose dependant increase in fluorescence with PMO-CP8M (Figs 10a and 10b). The lack of signal above background does not allow statistical analyses of the comparative increase in fluorescence, but clearly demonstrated that without the CP8M conjugation, PMO did not enter the cell.

**[00215]** Transfection experiments conducted in the HEK293T human embryonic kidney cells again demonstrated that PMO was refractory to cell entry. Importantly, a non-ring closed variant of CP8M (termed CP8M-NC) also did not result in significant cell entry above that of PMO alone. However a ring closed variant that contains the core sequence RKF-S5-RLF-S5 demonstrated that when conjugated to a fluorescently labelled PMO (to form PMO-C8M), that the PMO was now efficiently taken into cells (Fig 11). This confirms that the shape imposed

upon the peptide sequence, following a ring closing metathesis, is important to facilitate cellular entry of a DCCPM in which the BAC is an ON, more specifically a PMO.

**[00216]** The formal charge of CP8M is +3 at physiological pH. We also provide data demonstrating that reducing the formal charge within this sequence still leads to a surprising and significant cellular entry of CP8M variants with formal charges of +2 (CP8M-2) and +1 (CP8M-1) (Figs 12a and 12b), particularly at lower concentrations. The reduction in charge leads to solubility issues which account for the failure of a dose dependent increase, unlike that observed with CP8M. Alternative excipients or manipulations of the peptide as highlighted in Table 5 and Table 6 are likely to overcome these solubility issues.

**[00217]** When transfection experiments were conducted in the $H_2K$ mdx mouse myoblasts cells, it confirmed the finding that PMO are refractory to muscle cell entry, which was overcome with the conjugation of CP8M (Fig 13) and that the CP8M 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 (Fig 14). Again, the lack of exon exclusion from the PMO only samples precludes comparative statistical analyses; but highlights that exon exclusion, resultant from entry of a PMO, only occurs when the PMO is conjugated to CP8M.

**[00218]** In order to determine if CP8M 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-CP8M (805.75) and the naked PMO (762.25) with no statistical significant difference (n=4, p=0.863; Fig 15 and Fig 16). Thus it was determined that CP8M does not confer any steric hindrance to the biological activity of the PMO.

**[00219]** Systemic administrations of PMO-CP8M and PMO were conducted in *mdx* mice to determine if the CP8M moiety enhanced cell entry into skeletal muscle. Varying sub-optimal amounts (totally 1 μmol/kg, 4.4 μmol/kg, 10.9 μmol/kg) were administered by either intraperitoneal or intravenous injections, and diaphragm and/or *Tibialis anterior* (TA) muscle recovered 7 days post-administration. After a single intraperitoneal administration of 1 μmol/kg the diaphragm muscle gave more dystrophin positive fibres after PMO-CP8M treatment compared to the PMO control group (Fig 17a, n=1). In tibialis anterior muscles, intravenous (4.4 μmol/kg; n=3) data was equivocal between groups (Fig 17b and Fig 17d, p=0.201), as was the intraperitoneal (10.9 μmol/kg; n=4) data (Fig 17c and Fig 17d, p=0.886).

**[00220]** (4.4 μmol/kg; n=3) data was equivocal between group (Fig 17b and Fig 17d, p=0.201), as was the intraperitoneal (10.9 μmol/kg; n=4) data (Fig 17c and Fig 17d, p=0.886).

**[00221]** Systemic intraperitoneal administrations of PMO-CP8M and PMO were conducted in *mdx* mice to determine if CP8M enhanced cell entry (Figs 18-21). A series of

tissues was recovered and frozen 1 or 2 weeks post-administration (skeletal muscle, heart, brain and liver).

**[00222]** A single low dose of PMO or PMO-CP8M (1 µmol/kg) was administered (n=1 per group) and tissues recovered 7 days post-administration. Dystrophin-positive heart muscle fibres were detected after PMO-CPM8, but not PMO administration (Fig 18a).

**[00223]** In addition, we carried out repeated intraperitoneal administrations into *mdx* mice. Intraperitoneal injections of PMO-CP8M (without fluorescent label) totalling 7.6 µmol/kg over 4 days (n=4 per group) lead to a significant increase in dystrophin-positive heart muscle fibres 2 weeks post-administration compared to injection of an equimolar amount of PMO (340±69 vs 57±17 fibres, $p<0.01$; Figs 18b & 18d).

**[00224]** Furthermore, Applicant carried out intraperitoneal administration of PMO or PMO-CP8M, totalling 10.9 µmol/kg over 2 days (n=4 per group). Again, administration of PMO-CP8M lead to a significantly higher number of dystrophin-positive heart muscle fibres compared to administration of PMO (680±163 vs 33±8 fibres, $p<0.05$; Figs 18c & 18e).

**[00225]** PMO-peptide conjugates of both 8.7 kDa and 9.2 kDa were successfully delivered to heart muscle fibres as evidenced by the induction of dystrophin re-expression in the hearts of *mdx* mice (Fig 19, n=4).

**[00226]** A single low dose (1 µmol/kg) intravenous administration of PMO-CP8M, but not PMO (both without fluorescent label), led to recovery of dystrophin expression in the Purkinje cells of *mdx* mouse cerebellum (Fig 20; n=1 per group). No dystrophin expression was observed in the PMO control.

**[00227]** A single low dose (1 µmol/kg) intravenous administration of PMO-HP8M led to increased hepatocyte fluorescence in the vicinity of blood vessels two weeks post-administration compared to administration of PMO (Fig 21; n=1 per group), implying increased uptake of PMO-HP8M in hepatocytes.

**Conclusion**

**[00228]** From the data generated 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 into a cell. The StaP CPA facilitated PMO entry in both *in vitro* and *in vivo* assay systems.

**[00229]** Applicant's data presents evidence that modified linker systems based on HNA and 4 formyl benzioic acid improve the efficiency of conjugation between a BAC and CPA.

**[00230]** Surprisingly, variants of CPA in which the formal charge is reduced demonstrate enhanced cell entry at lower concentrations. This will have important sequelae with respect to improving the toxicological profile of CPA, more specifically a CPP.

**[00231]** 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.

**[00232]** The data demonstrates the fact that in the *in vivo* model of RNA splicing suppression the stabilized CPA may enhance cell entry into skeletal muscle, particularly at lower doses. However PMO are known to enter skeletal muscle without a CPP conjugation.

**[00233]** 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.

**[00234]** Applicant further provides evidence that the CPP can facilitate the entry of cargoes of different size and mass far beyond that stated in the current state of the art.

**[00235]** 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).

2. A DCCPM as claimed in claim 1 wherein the StiP or StaP comprises a cross link or bridge between two amino acids of the peptide.

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

4. A DCCPM as claimed in claim 2 wherein the peptide comprises at least two un-natural amino acids bearing all-hydrocarbon tethers (*e.g.* α-methyl, α-pentenyl glycine).

5. A DCCPM as claimed in claim 3 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**).

6. A DCCPM as claimed in claim 2 comprising a cross link or bridge between one or more of the unnatural amino acids of Table 1.

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

8. A DCCPM as claimed in any of the preceding claims wherein the stabilized conformation comprises at least one alpha helix.

9. A DCCPM as claimed in any of the preceding claims wherein the stabilized conformation comprises at least one beta sheet.

10. A DCCPM as claimed in any of the preceding claims wherein the stabilized conformation comprises at least one alpha helix and one beta sheet.

11. A DCCPM as claimed in any of the preceding claims wherein the BAC is an oligonucleotide (ON).

12. A DCCPM as claimed in claim 11 wherein the ON is an electrically low charge carrying oligonucleotide.

13. A DCCPM as claimed in claim 12 wherein the ON is an electrically neutral charge carrying oligonucleotide

14. A DCCPM as claimed in claim 12 or 13 wherein the ON is a polynucleic acid (PNA) or a phosphorodiamidate morpholino oligonucleotide (PMO).

15. A DCCPM as claimed in any of claims 11 to 14 wherein the ON is an anti-sense oligonucleotide (AON).

16. A DCCPM as claimed in claim 15 wherein the ON is an anti-sense oligonucleotide comprising a chemistry selected from the compounds of Table 4.

17. A DCCPM as claimed in claim 11 wherein the BAC is a phosphorodiamidate morpholino oligonucleotide (PMO).

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

19. A DCCPM as claimed in claim 18 wherein the endogenous gene targets a neuromuscular disease, a metabolic disease, cancer, an age-related degenerative disease or an acquired viral infection.

20. A DCCPM as claimed in any of the preceding claims wherein the BFL comprises a chemistry selected from the chemistries of Table 5.

21. A DCCPM as claimed in claim 20 comprising an amine to sulphydryl cross linker containing N- hydroxysuccinimide esters and malemide reactive groups separated by a cyclohexane spacer.

22. A DCCPM as claimed in claim 20 or 21 wherein the BFL is a succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC).

23. A DCCPM as claimed in claim 1 wherein the BFL is HNA and has been incorporated into the terminal end of the CPP.

24. A DCCPM as claimed in claim 23 wherein the BFL is HNA and the ON has been modified to incorporate 4 formyl benzioic acid to facilitate covalent conjugation.

25. A DCCPM as claimed in claim 22 wherein the SMCC is PEGylated.

26. A DCCPM as claimed in claim 23 of 24 wherein the HNA is PEGylated

27. A DCCPM as claimed in claim 20 wherein the CPA is linked to a first end of the BFL covalently.

28. A DCCPM as claimed in claim 20 wherein the BAC is linked to a second end of the BFL covalently.

29. DCCPM as claimed in claim 1 which is of a size greater than 1.5KDa.

30. A method for facilitating the uptake of a biologically active compound (BAC) into a cell by the conjugation of the biologically active compound to a cell penetrating agent (CPA) which is a stabilized peptide which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP) 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.

31. A DCCPM as claimed in any of claims 1-29 for use in the treatment of a disease requiring alteration of the expression of an endogenous or exogenous gene.

32. A DCCPM as claimed in claim 31 for use in the treatment of neuromuscular disease, a metabolic disease, cancer, an age-related degenerative disease or an acquired viral infection.

33. A DCCPM as claimed in claim 31 for use in the treatment of Duchenne’s muscular dystrophy.

34. A DCCPM as claimed in claim 33 wherein the DCCPM comprises an AON targeting exon 51 of the dystrophin gene.

35. A method of improving the bioavailability of a drug or BAC comprising linking the drug or BAC to a CPP which is a stabilized peptide which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP).

36. A method of introducing a drug or BAC to a site which is refractory to the drug or BAC in its native state comprising linking the drug or BAC to a CPP which is a stabilized peptide which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP) and administering it to a subject..

37. A method as claimed in claim 36 wherein the tissue is one of heart, brain, muscle or liver.

38. A method of treating a subject to alter the expression of an endogenous or exogenous gene comprising administering a DCCPM as claimed in any of claims 1-29 or 31-34.

39. A composition comprising a DCCPM as claimed in any of claims 1-29 or 31-34 and one or more pharmaceutically acceptable excipients.

40. A composition as claimed in claim 39 which is adapted for administration orally, parenterally, intravenously or topically

ABSTRACT

The invention relates to improvements in drug delivery and more particularly to the use of Cell Penetrating Agents (CPA's) or 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).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). The preferred BAC is an electrically low charge carrying oligonucleotide such as a phosphorodiamidate morpholino oligonucleotide (PMO). 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.