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(71) Applicant(s):
Sutura Therapeutics Ltd
(Incorporated in Turks and Caicos)
Suites A201 & A202 Regent Village East, Grace Bay, Providenciales, Turks and Caicos

(72) Inventor(s):
Keith Foster
Adam James Reginald Gadd

(74) Agent and/or Address for Service:
Equipped 4 (IP) Limited
47 Hamilton Square, BIRKENHEAD, Merseyside, CH41 5AR, United Kingdom

(51) INT CL:
A61K 47/64 (2017.01) ***A61P 21/00*** (2006.01)
C07K 1/107 (2006.01)

(56) Documents Cited:
WO 2017/011820 A2 **WO 2016/187425 A1**
"Stitched alpha-helical peptides via bis ring closing metathesis", G.J. Hilinski et al, J. Am. Chem. Soc., (2014), 136, 12314-12322

(58) Field of Search:
INT CL **A61K**
Other: **BIOSIS, CAS-ONLINE, MEDLINE, PATENT FULLTEXT & WPI**

(54) Title of the Invention: **Improvements in drug delivery**
Abstract Title: **Drug delivery using stabilised cell penetrating peptides**

(57) Improvements in drug delivery are brought about by Cell Penetrating Agents (CPA's) or Cell Penetrating Peptides (CPP's) which have been stabilized by, for example: stapling two amino acids to form Stapled CPP's (StaP's) or stitching three or more amino acids to form stitched CPP's (StiP's). More particularly there is provided a drug carrying cell penetrating molecule (DCCPM) comprising: a biologically active compound (BAC), and a cell penetrating agent (CPA), which BAC and CPA are linked directly or via a bi-functional linker (BFL). The CPA is a stabilized peptide (CPP) which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP). The StiP or StaP comprise a cross link or bridge between at least two amino acids of the peptide and the cross link or bridge provides a cyclisation between at least two amino acids which are not formed by an olefin metathesis. Cyclisation may be achieved by one or more of: condensation of an aldehyde or ketone with a hydrazine or protected hydrazine; a thiol-ene Michael addition; a di-sulfide formation; a Huisgen 1,3 di-polar cycloaddition; a reaction between an amine and carboxylic acid; a singlet or triplet based carbine reaction; or a Suzuki or Sonogashira coupling.

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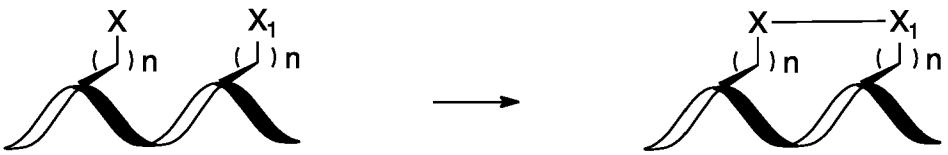
Fig 1a:



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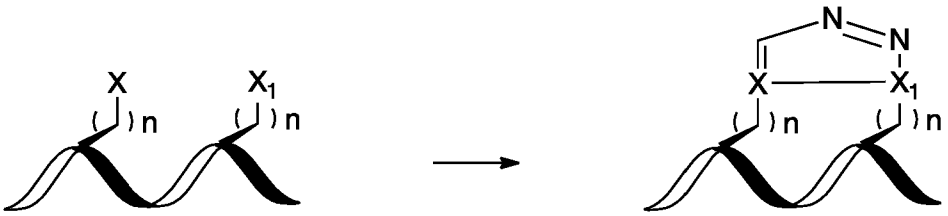

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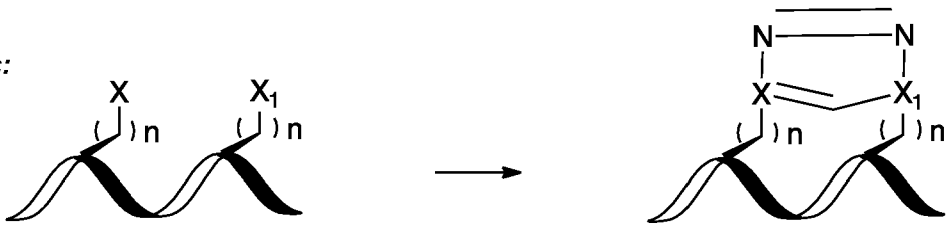

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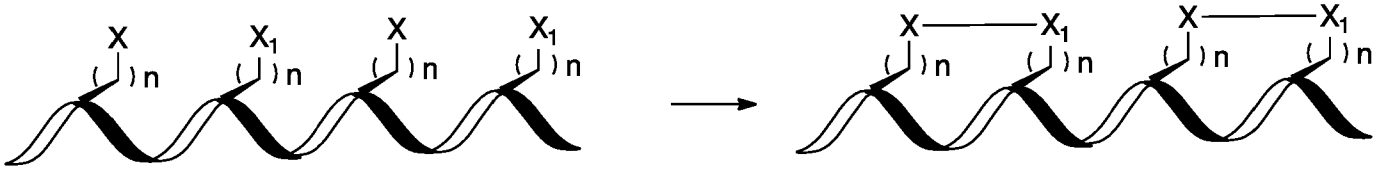

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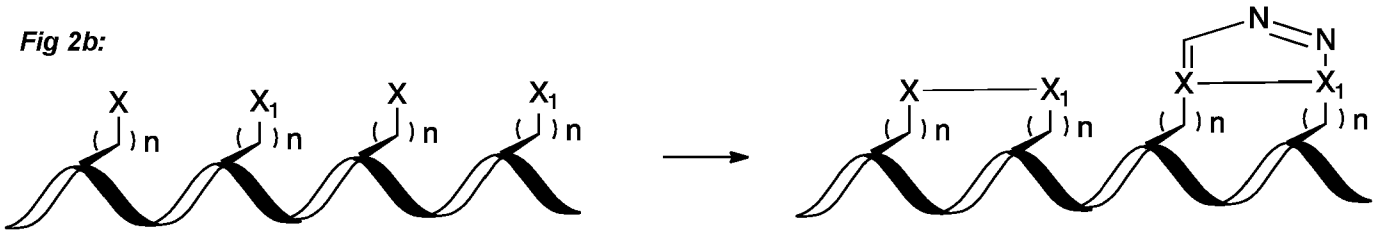

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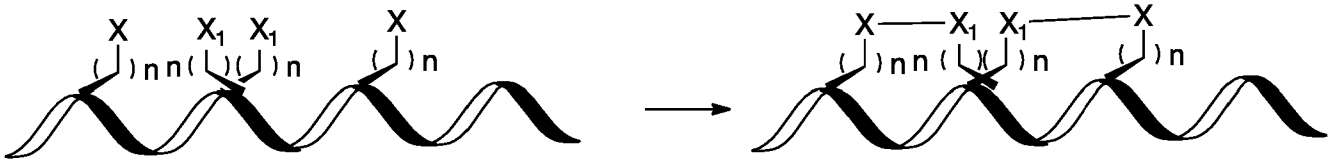

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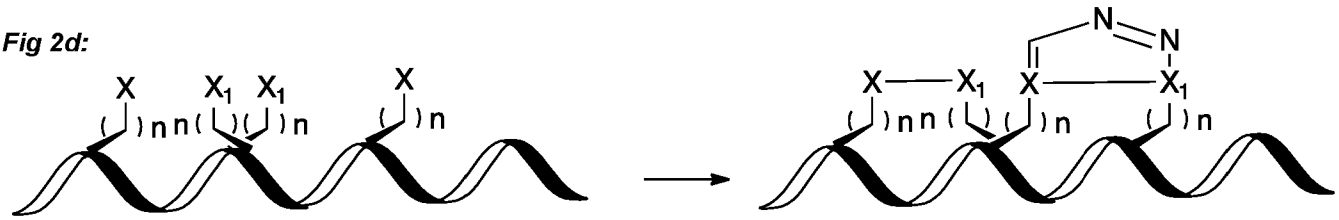

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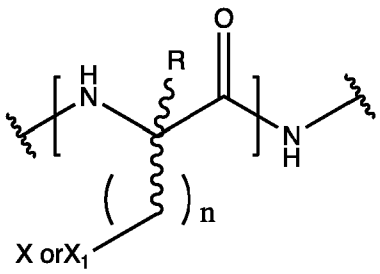

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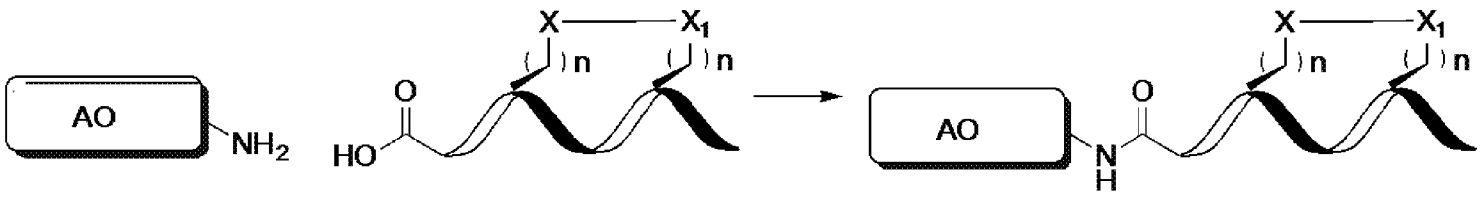

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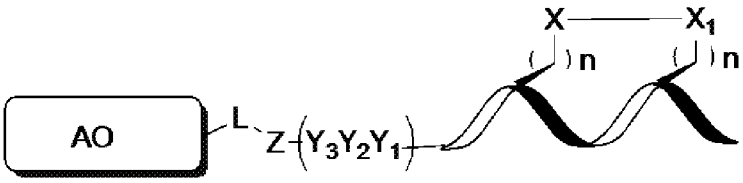

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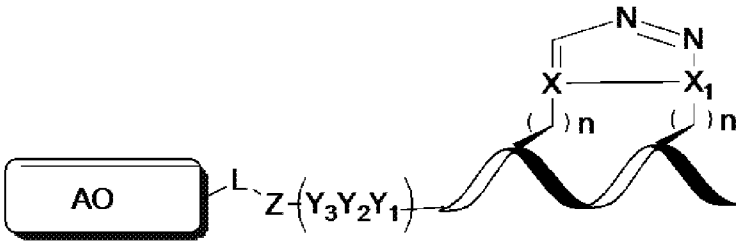

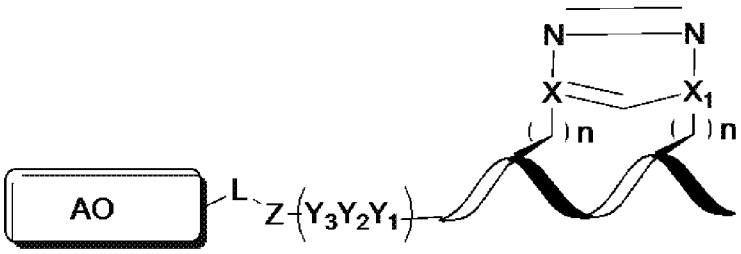

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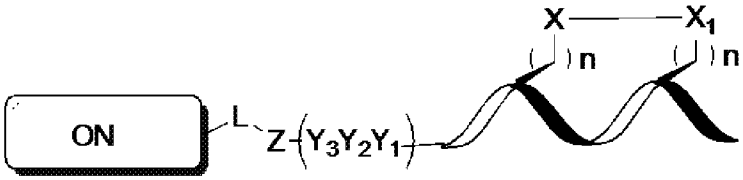

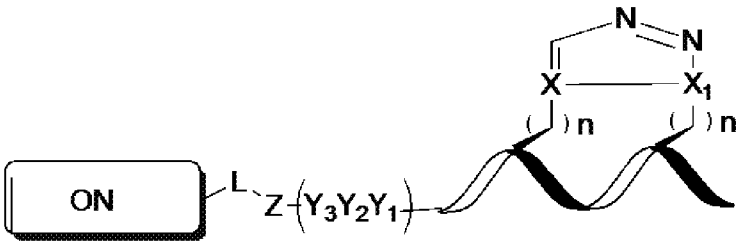

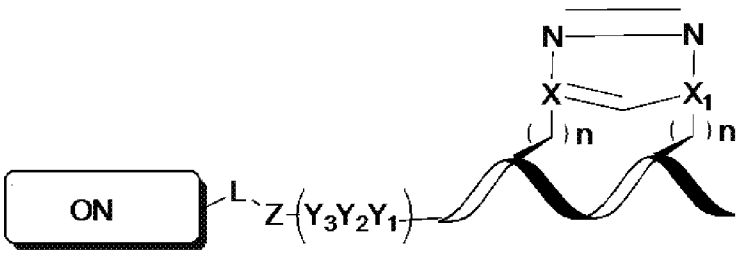

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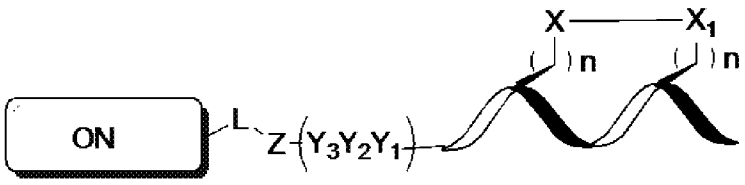

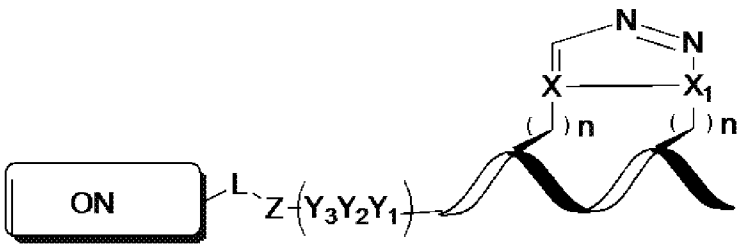

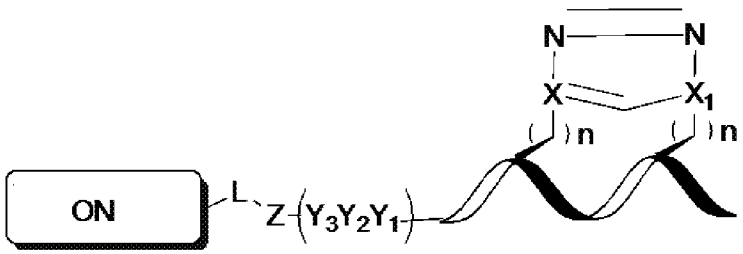

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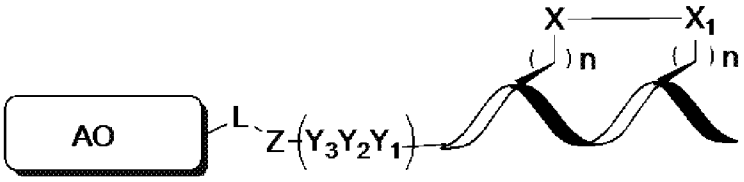

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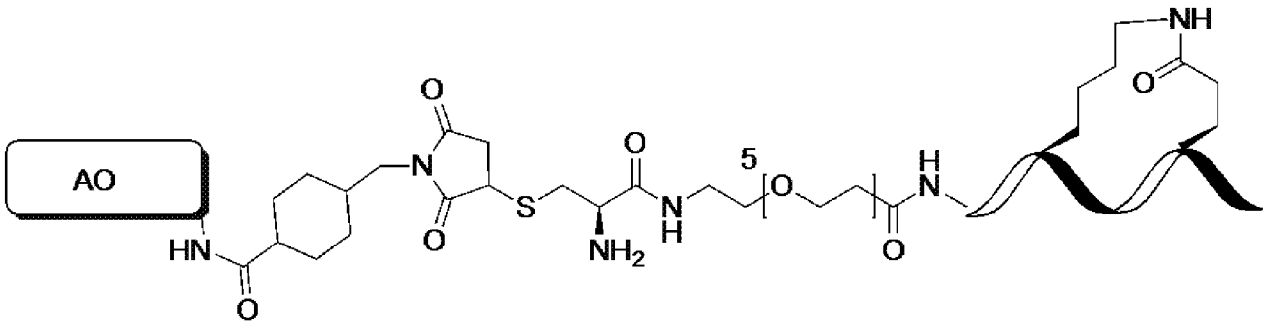

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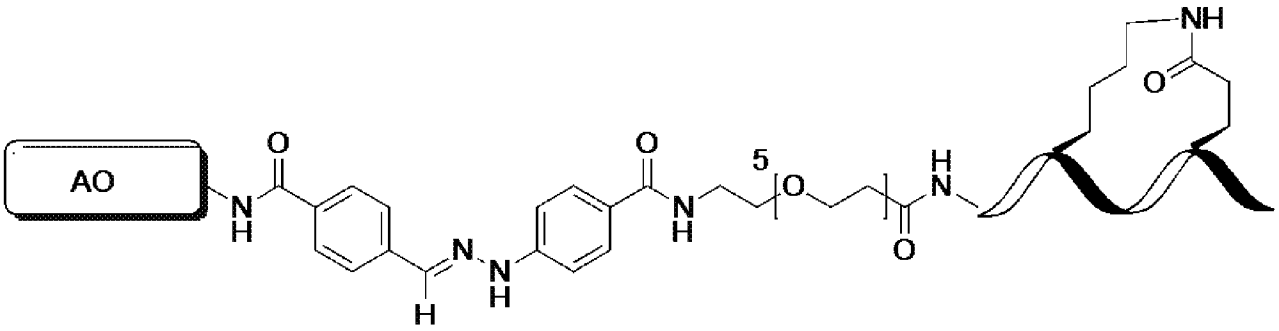

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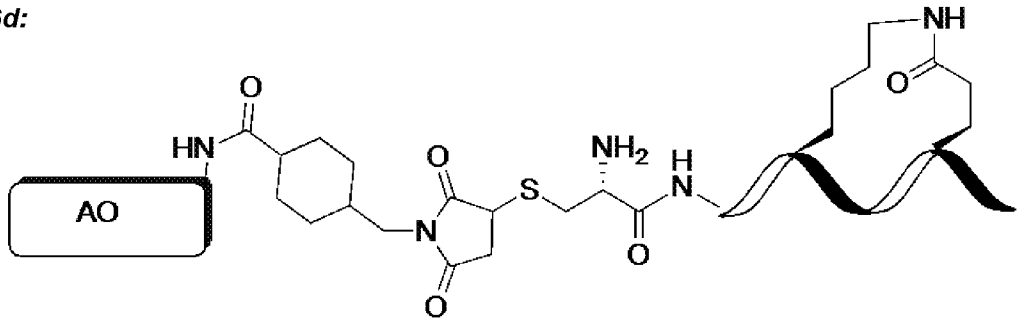

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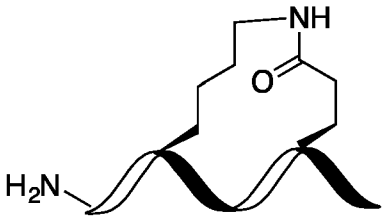

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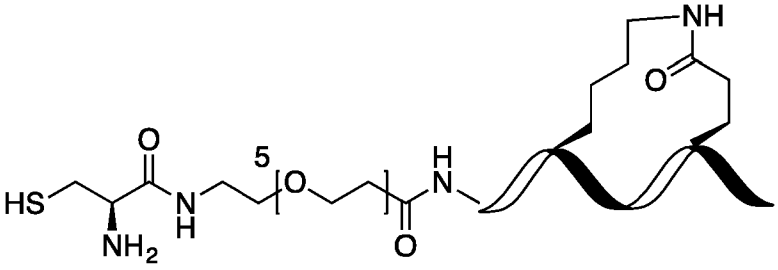

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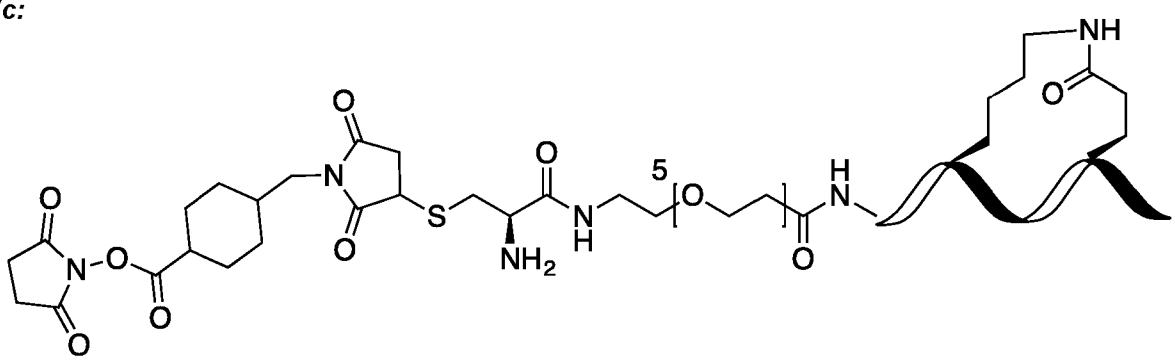

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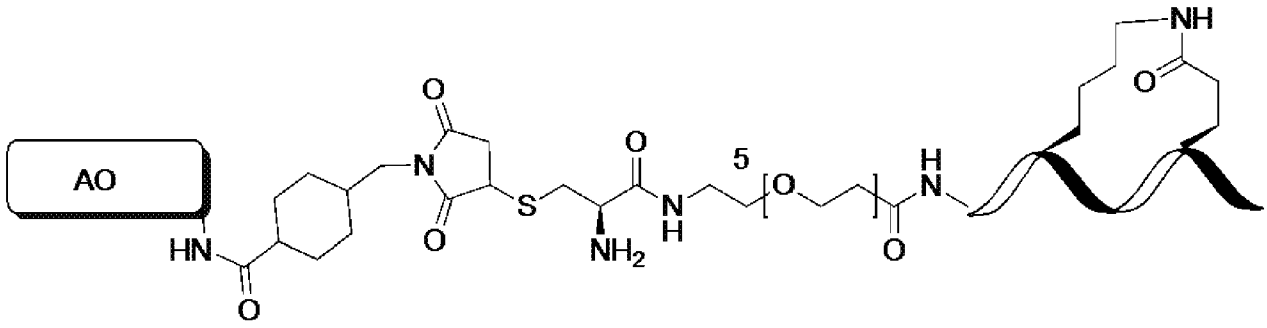

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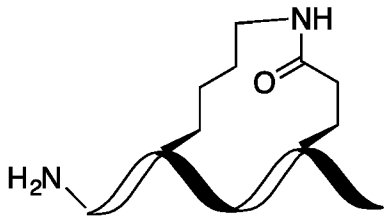

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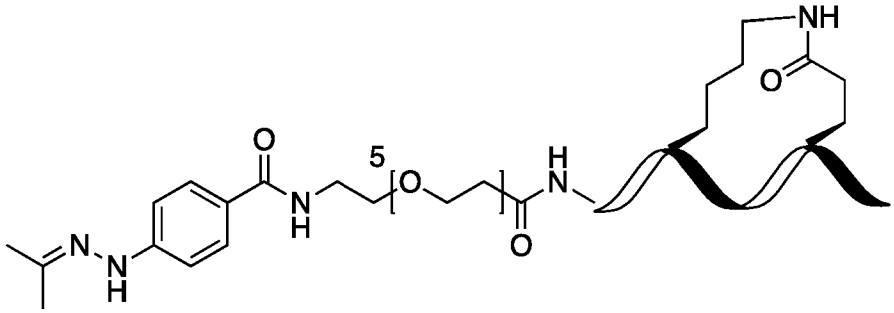

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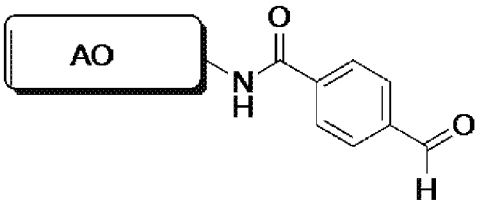

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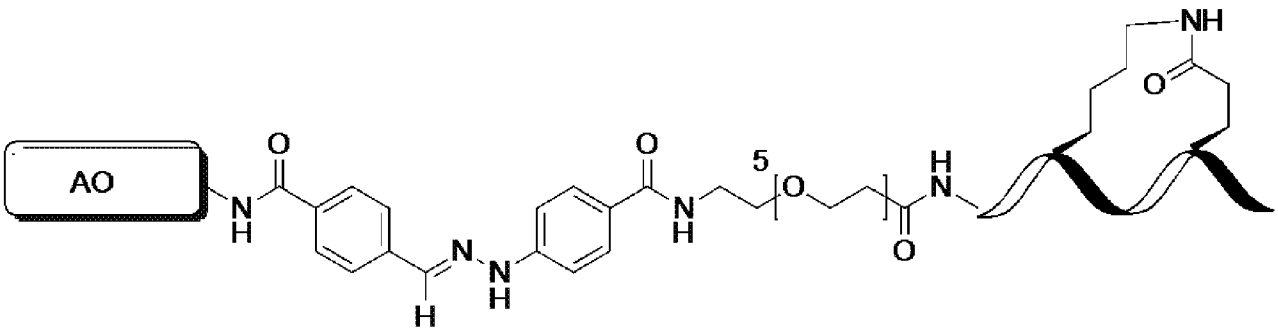
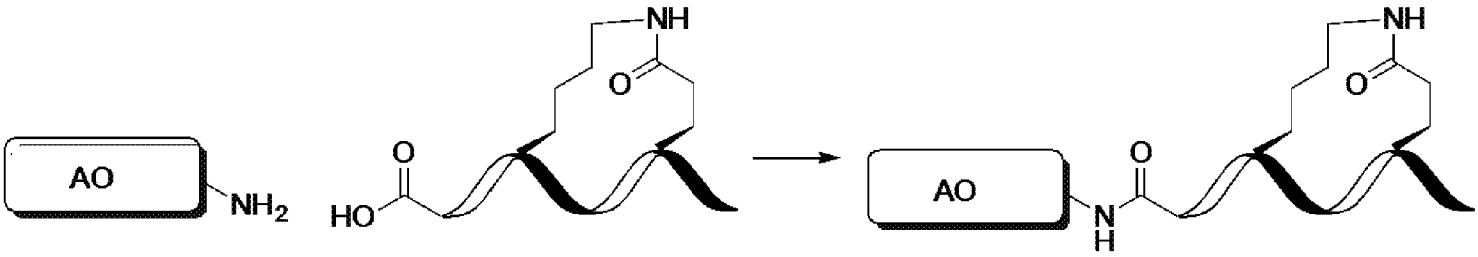


Fig 9



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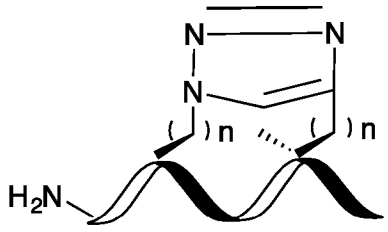

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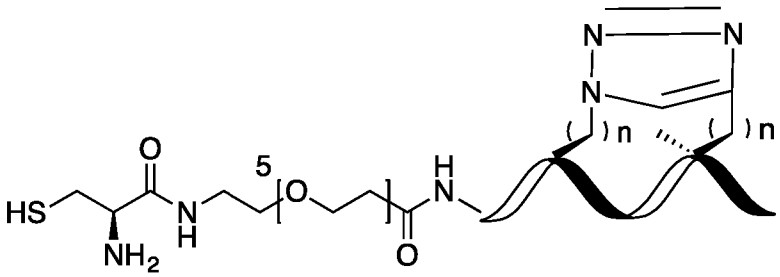

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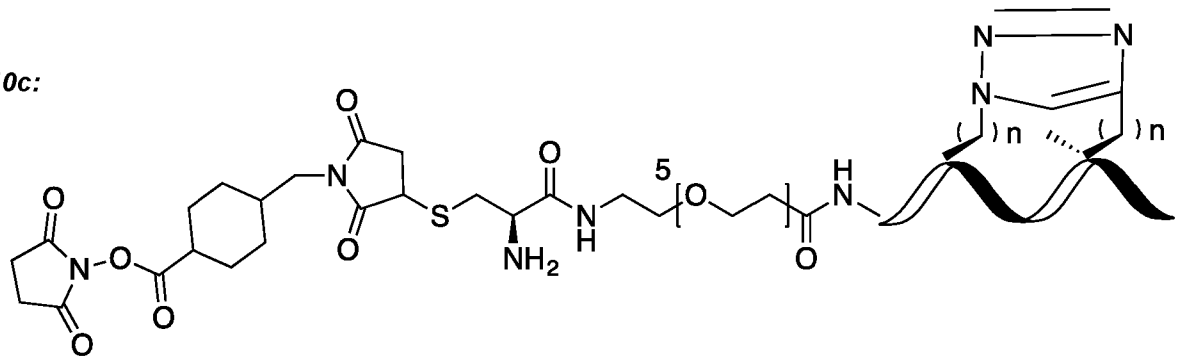

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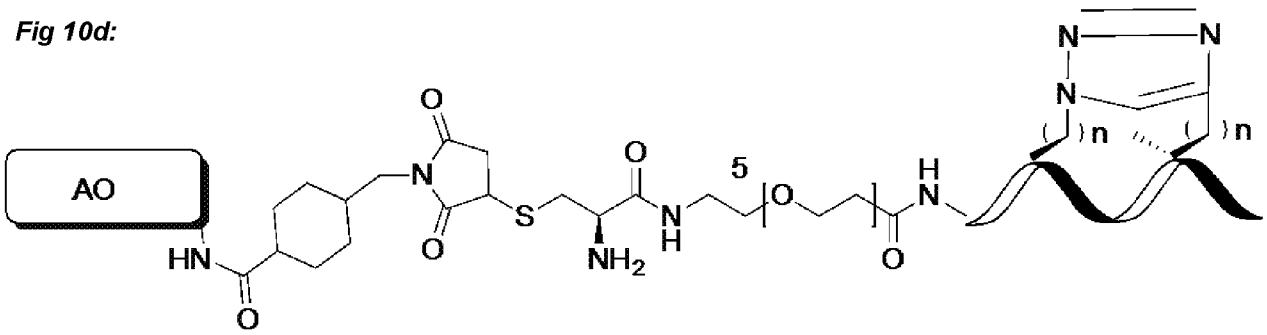

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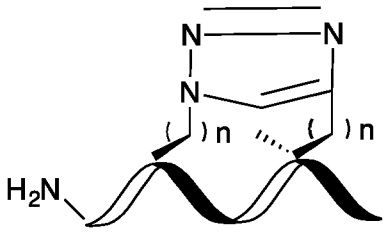

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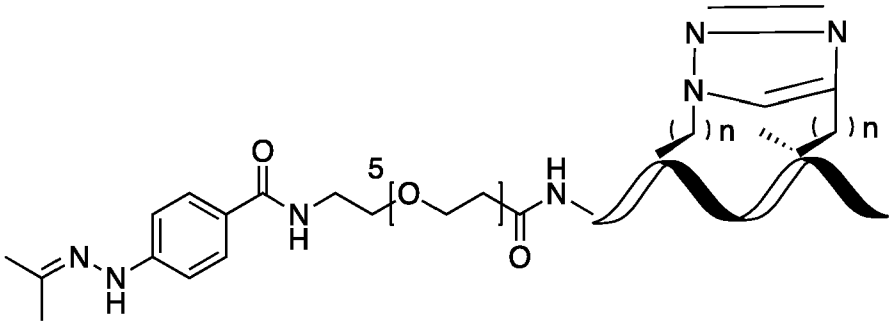

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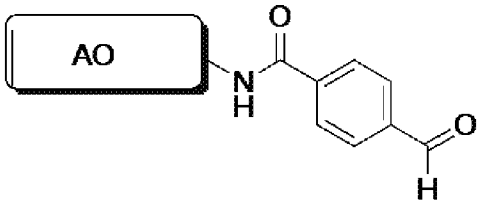

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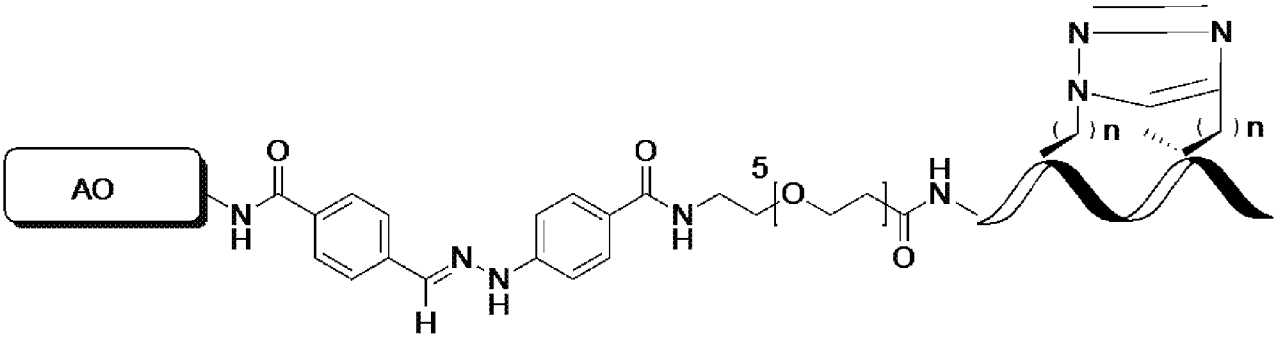

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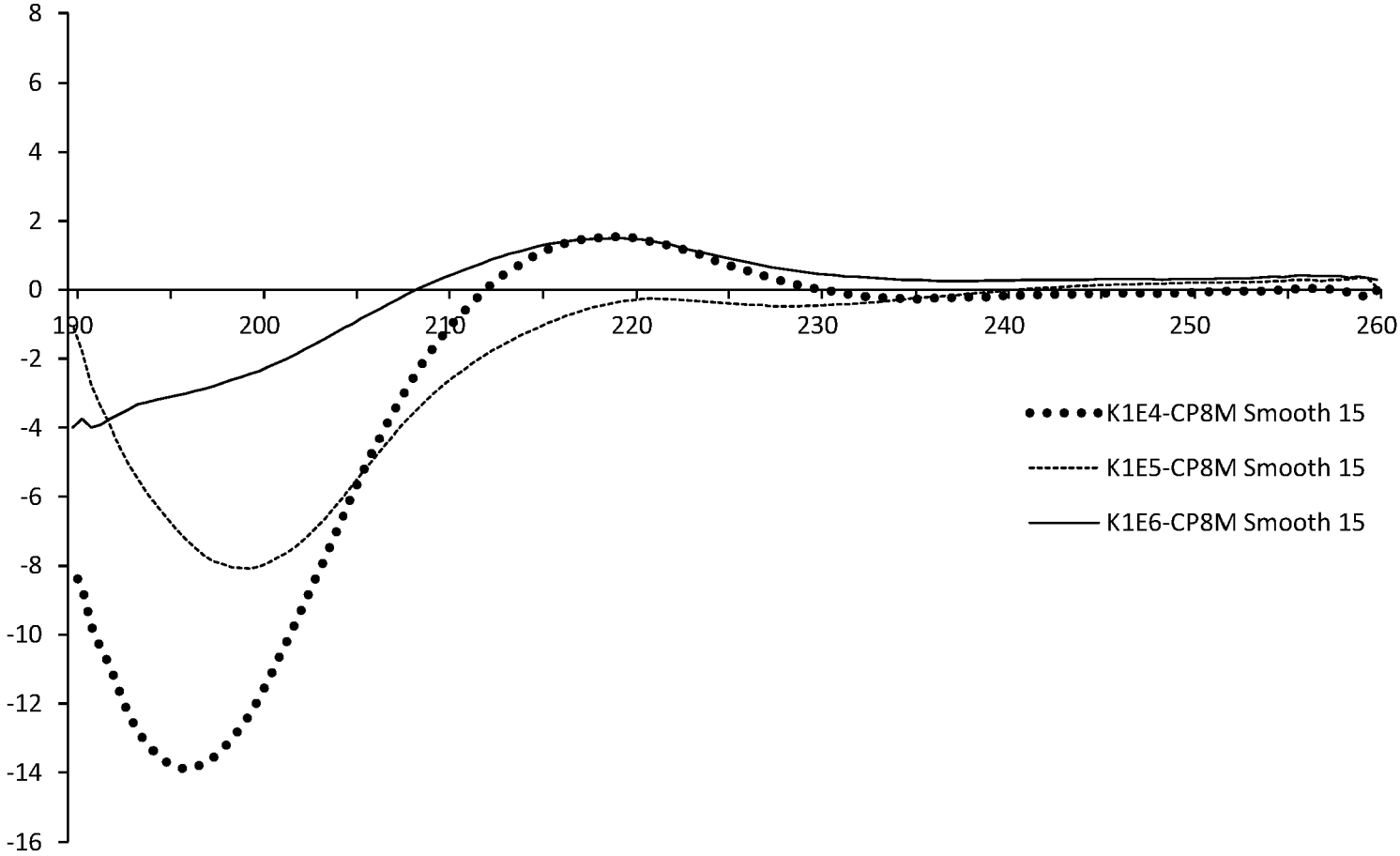
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200
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240
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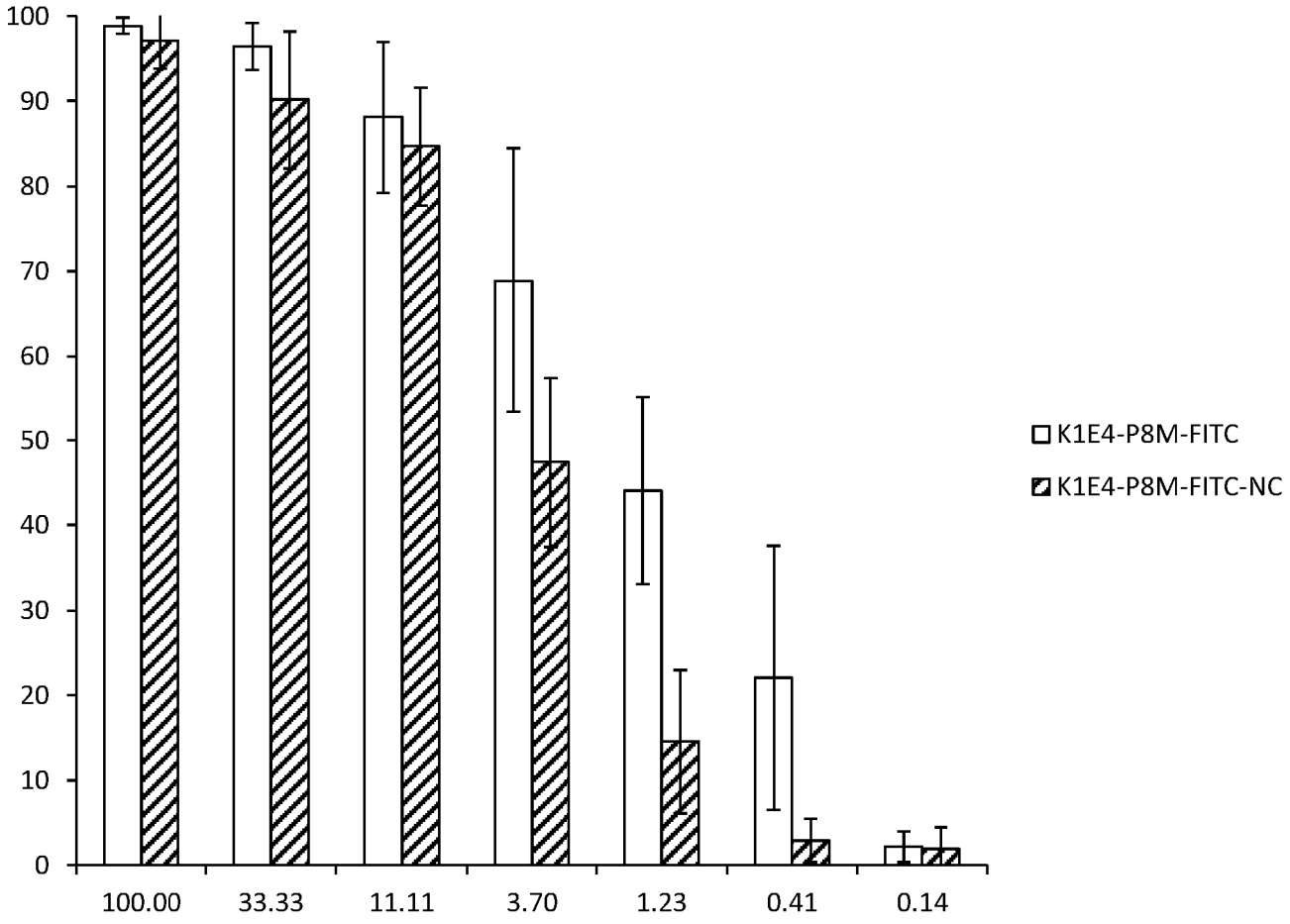
K1E6-CP8M Smooth 15

Fig 13ai



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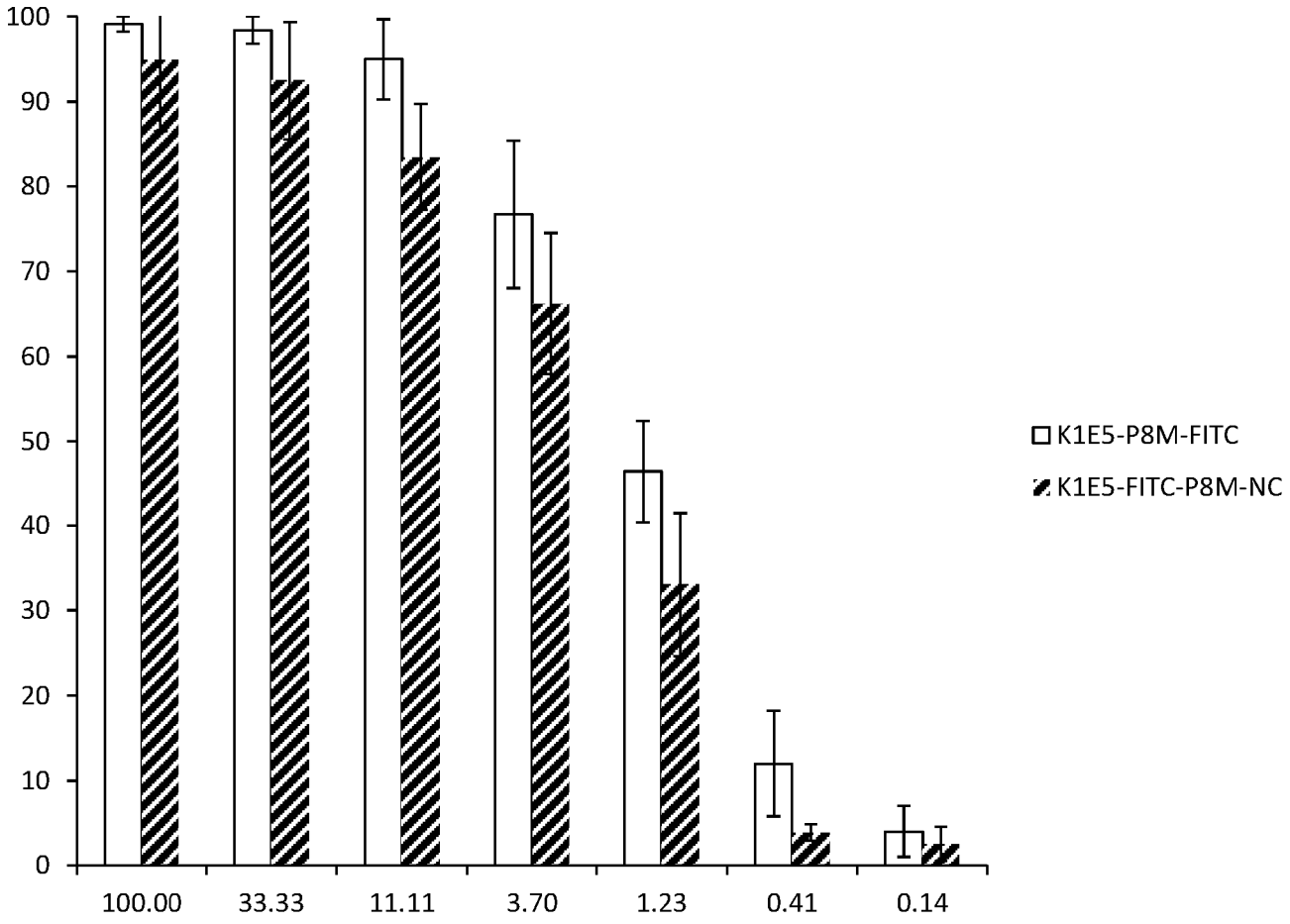

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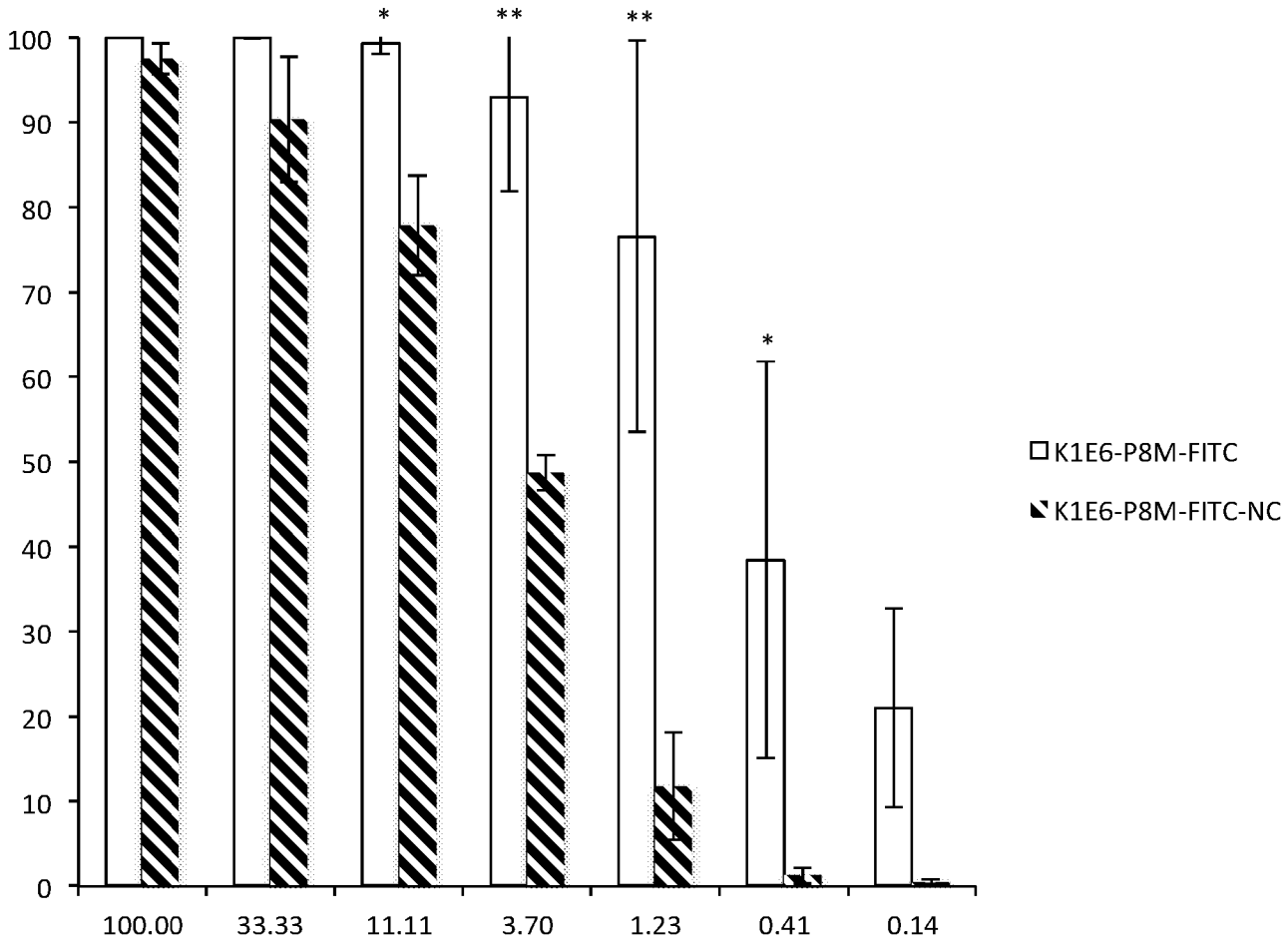

29.08.18

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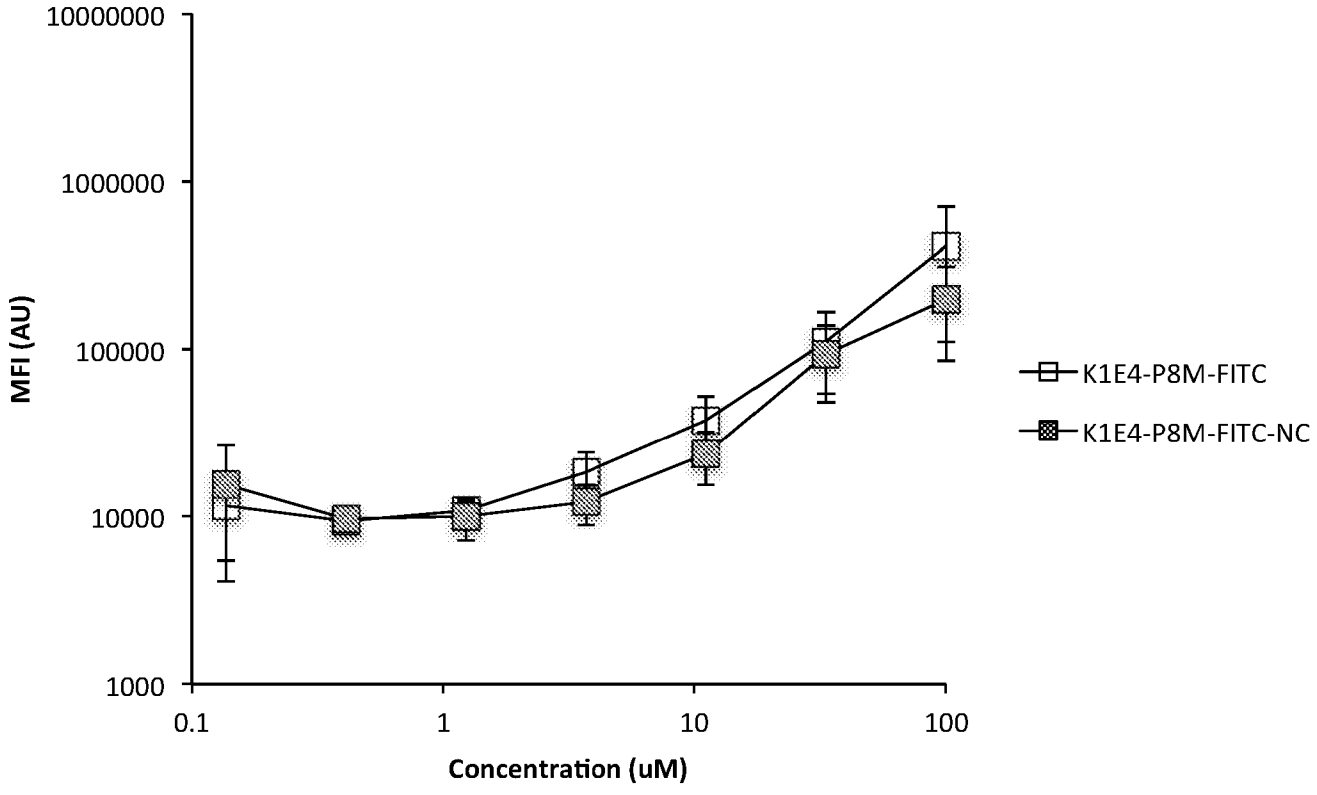

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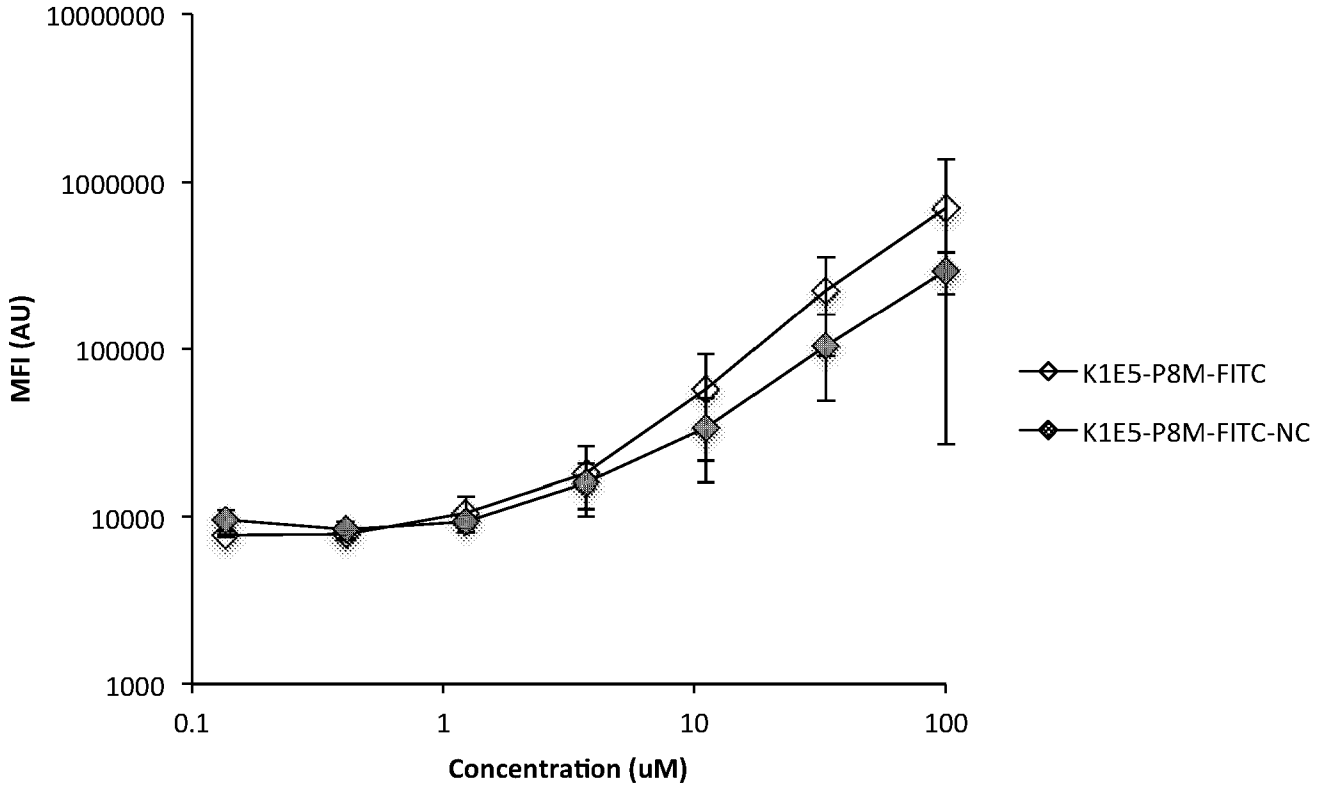

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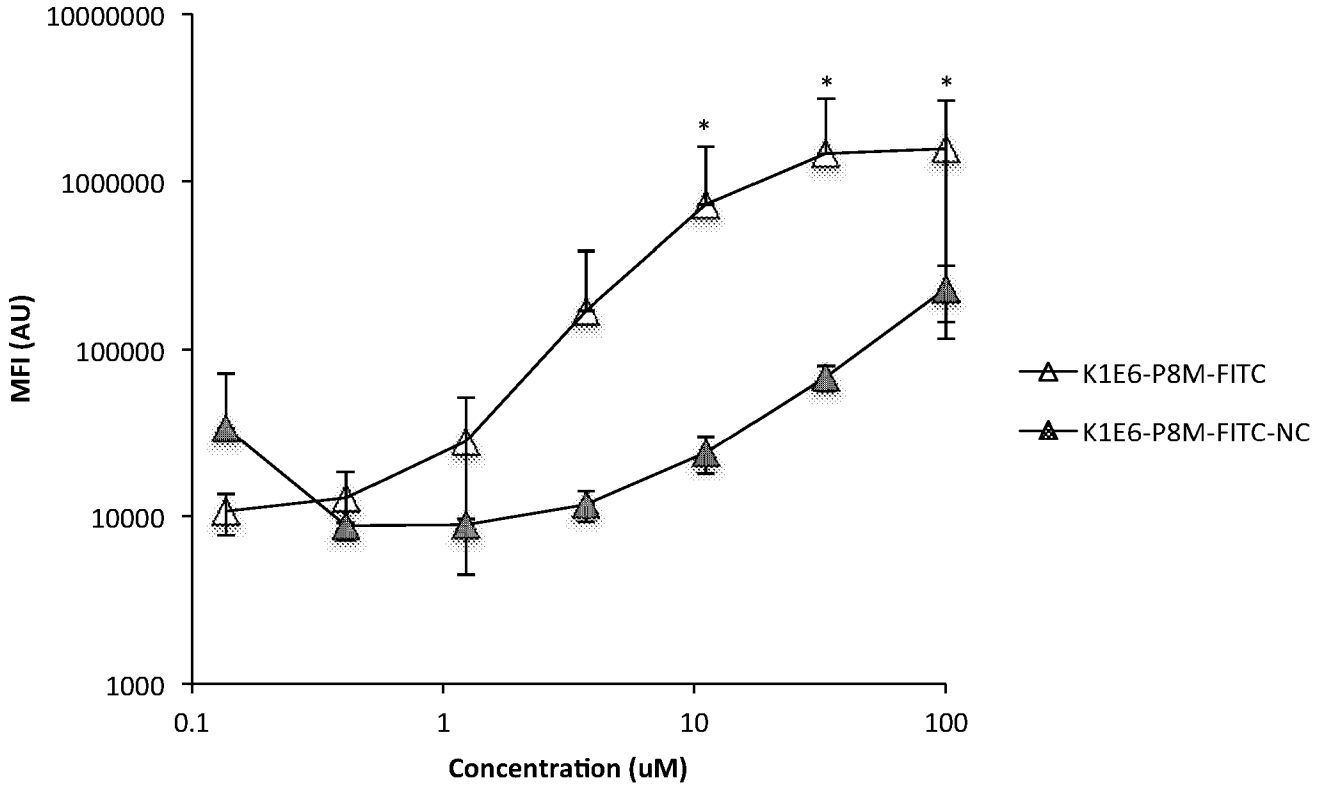

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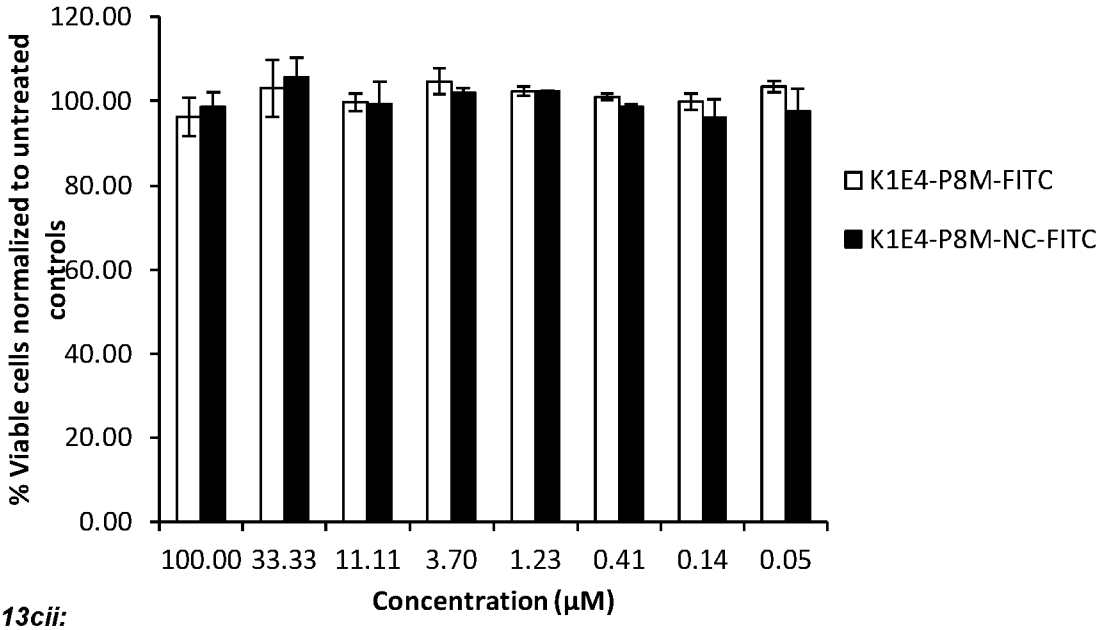

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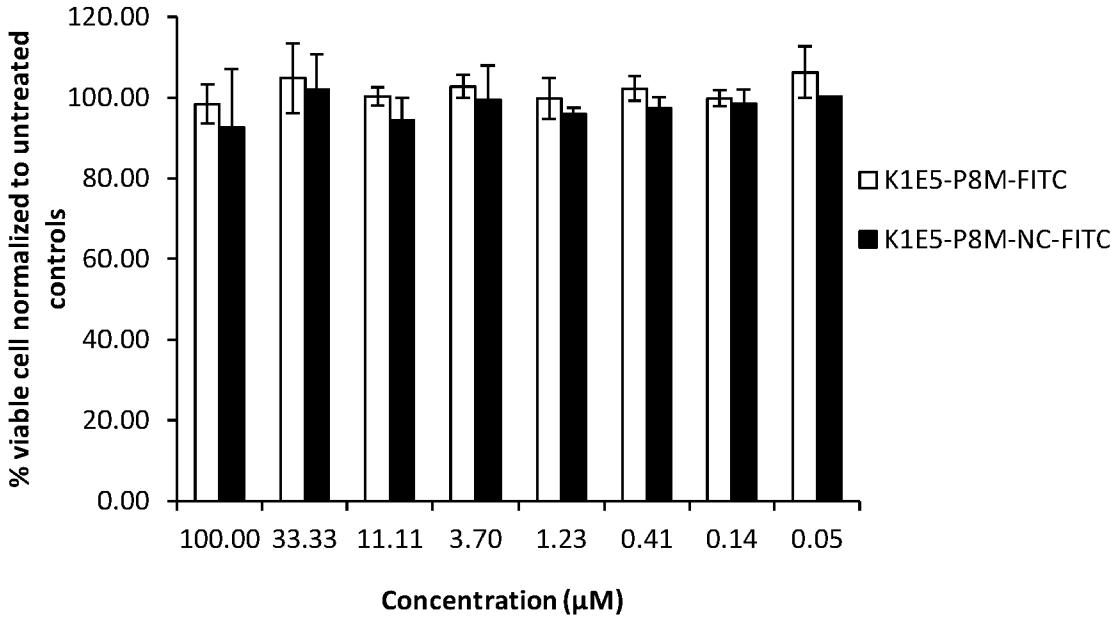

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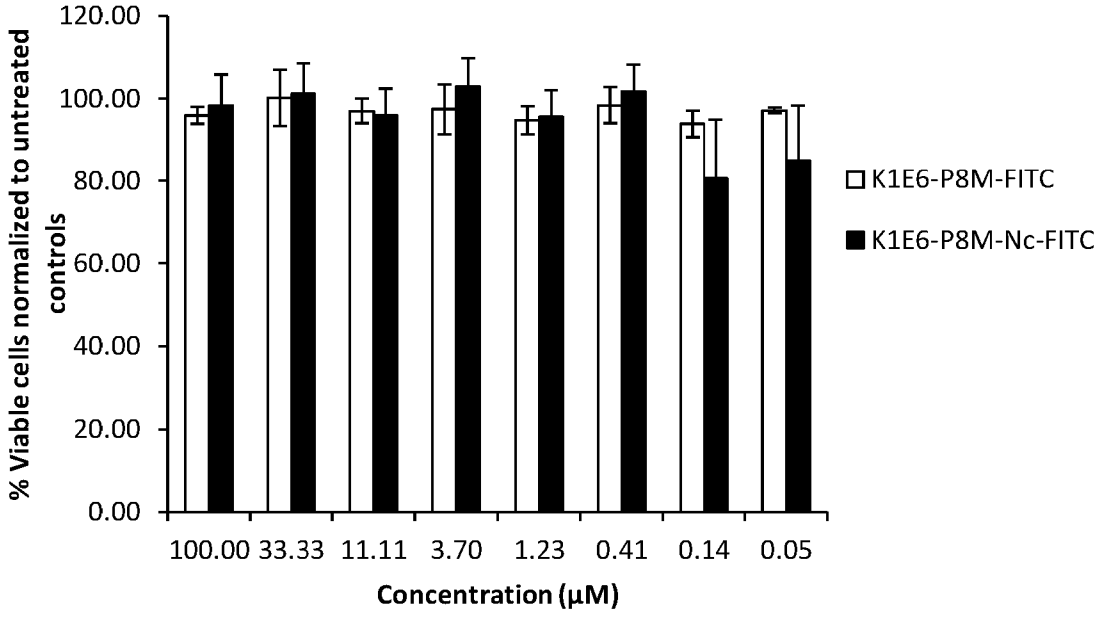

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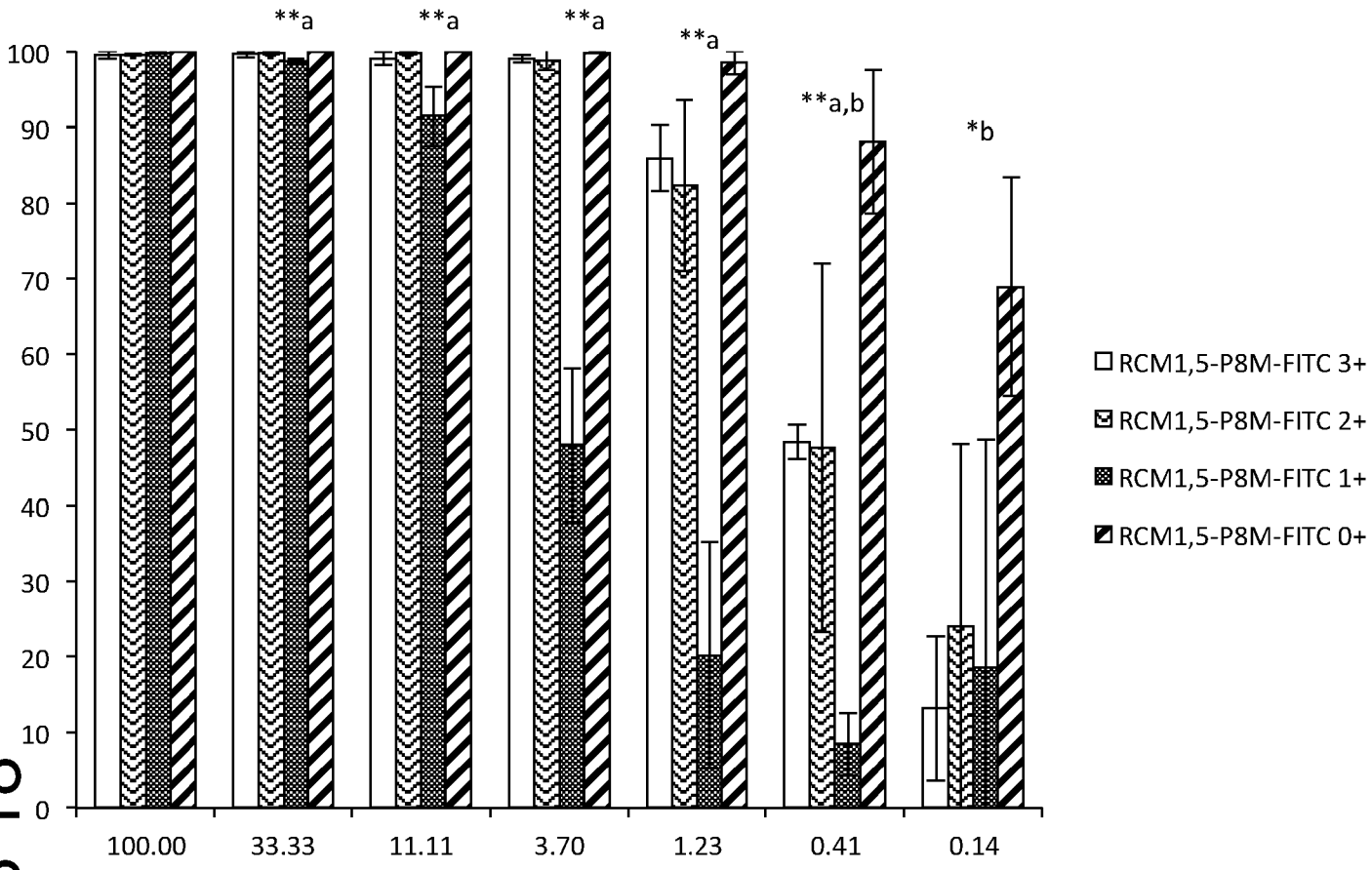

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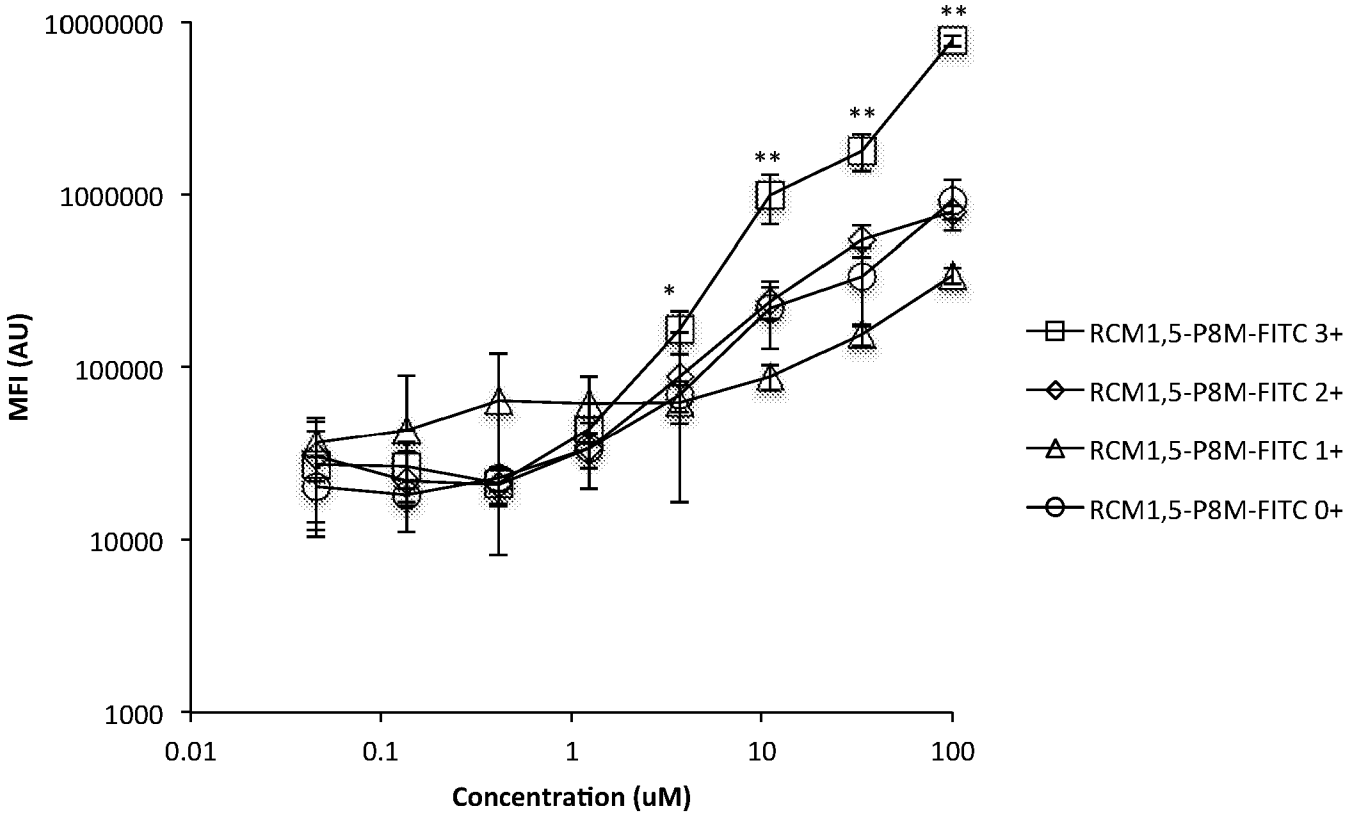

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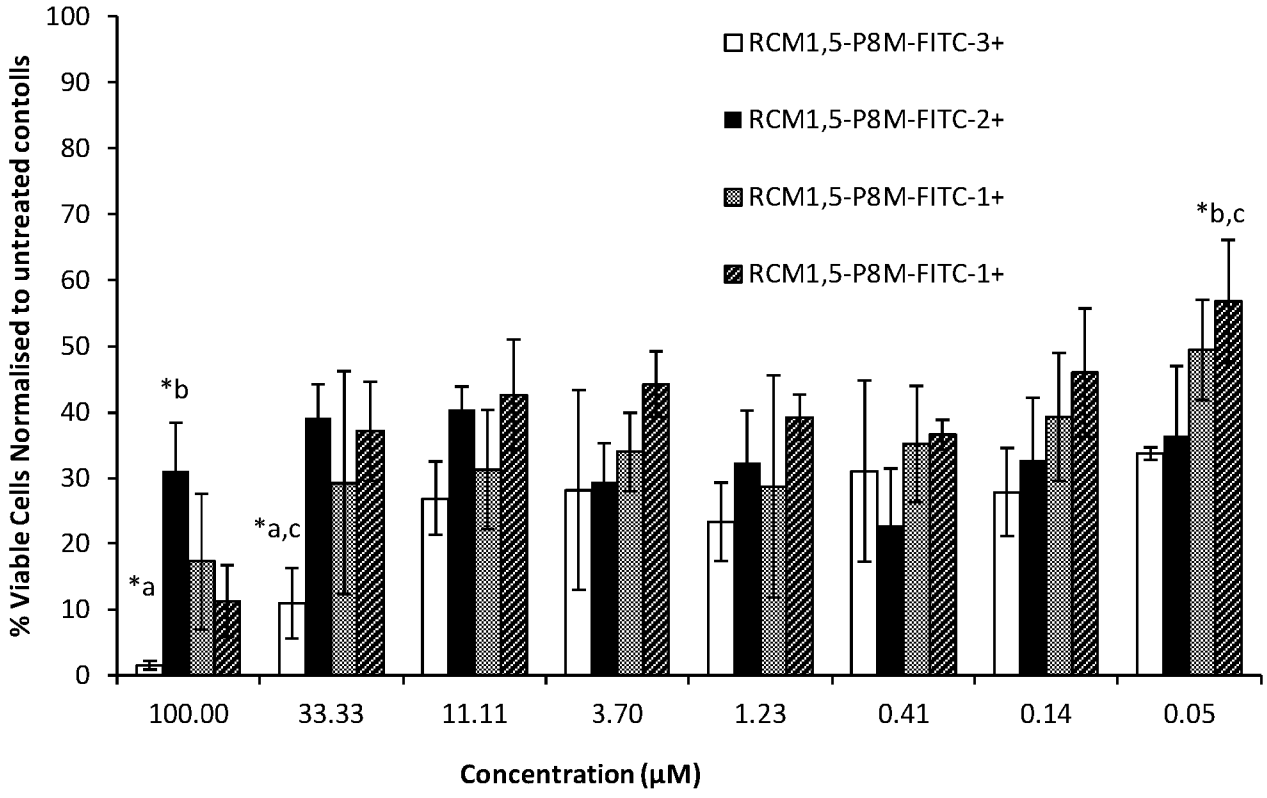
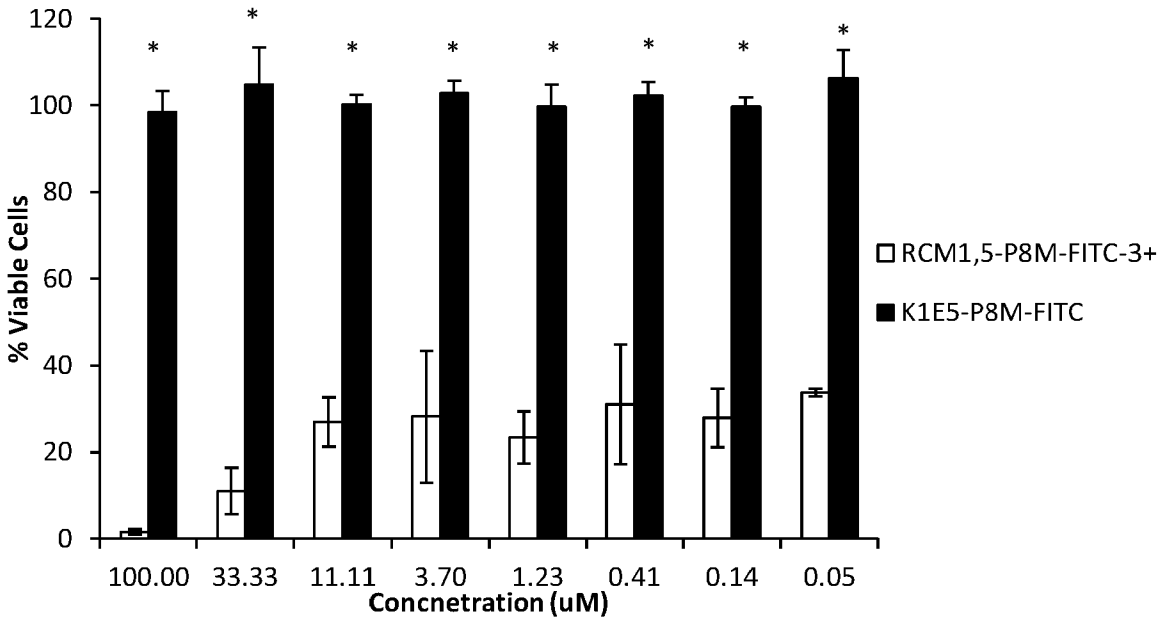

% Viable Cells Normalised to untreated contolls
100
90
80
70
60
50
40
30
20
10
0
RCM1,5-P8M-FITC-3+
RCM1,5-P8M-FITC-2+
RCM1,5-P8M-FITC-1+
RCM1,5-P8M-FITC-1+
*a
*b
*a,c
*b,c
100.00
33.33
11.11
3.70
1.23
0.41
0.14
0.05
Concentration (µM)

Fig 15:


120
100
80
60
40
20
0
% Viable Cells
*
100.00
33.33
11.11
3.70
1.23
0.41
0.14
0.05
Concnetration (uM)
RCM1,5-P8M-FITC-3+
K1E5-P8M-FITC

The following terms are registered trade marks and should be read as such wherever they occur in this document:

Penetratin p.4

Agilent p.29

Speedvac p.29

Orbitrap p.31

Chirascan p.31

milliQ p.31

sephadex p.32

FlowJo p.33

Kinetex p.33

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] It differs from applicant's earlier patent application PCT/GB2016/054028 in using different chemistries to an olefin metathesis to stabilize the peptide. These chemistries provide a cross-link or bridge between at least two amino acids of the peptide, and the cross-link or bridge provides a cyclisation between the at least two amino acids. In these alternative chemistries cyclisation is achieved by one or more of:

i. condensation of an aldehyde or ketone with a hydrazine or protected hydrazine;
ii. a thiol-ene Michael addition;
iii. a di-sulfide formation;
iv. a Huisgen 1, 3 di-polar cycloaddition;
v. a reaction between an amine and carboxylic acid;
vi. a singlet or triplet based carbine reaction; or
vii. a Suzuki or Sonogashira coupling.

[0004] These StaP's or StiP's may be composed of single or multiple staples or stitches and may be contiguous or non-contiguous along a peptide sequence.

[0005] 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).

[0006] 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, peptide nucleic acids (PNAs), phosphorodiamidate morpholino oligonucleotides (PMO's) or modified derivatives thereof.

[0007] Alternatively, stabilised CPP's when conjugated to an ON, either directly or via a BFL, can associate covalently or non-covalently with a BAC that is either a DNA, RNA or protein molecule so that the BAC can be carried though a cell membrane by the CPP conjugated ON. The resulting molecules are referred to as Drug Targeting Cell Penetrating Molecules (DTCPM's).

[0008] 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.

[0009] 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.

[0010] The invention also relates to a method of facilitating the uptake of a BAC that is either a DNA, RNA or protein molecule into a cell, the use of a DTCPM 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 DTCPM's of the invention and to a pharmaceutical composition comprising the DTCPM and one or more pharmaceutically acceptable excipients.

[0011] Still further aspects will be apparent from the detailed description.

BACKGROUND TO THE INVENTION

[0012] 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.

[0013] An example of drugs or BACs which are delivered in a targeted manner are oligonucleotides (ON's), which term includes ON analogues.

[0014] 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.

[0015] 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. Similarly, ON's can alter cellular protein content via modulating RNA splicing to exclude specific exons of a gene from the mature mRNA transcript. Together, this has led to translational programs for conditions such as Duchenne muscular dystrophy (exon exclusion) and spinal muscular atrophy (exon inclusion)[1,2] and subsequent market approval.

[0016] 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 (AO) to suppress viral translation is a well-established technology[3] and has progressed into clinical trials for viral haemorrhagic fevers such as Marburg and Ebola[4,5]. Although, AO can be designed to target 5' translations initiation starts sites of endogenously expressed genes.

[0017] Also, ON can be designed to target 3' untranslated region of an endogenous transcript that alters the nuclear export, translation and stability of the transcript. Such targets include, and are not limited to, poly adenylation and/or cleavage sites of the transcript.

[0018] 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).

[0019] 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.

[0020] Modifications of an ON to produce a negatively charged backbone improve stability[6-9], but these backbone chemistries *e.g.* 2'O-Methyl Phosphothioate analogues, elicit membrane toxicity issues, cause thrombocytopaenia and injection site problems upon clinical translation[10], such that efficacy is prevented by toxicity issues, even when administration protocols become increasingly intermittent[11].

[0021] Indeed WO2013/150338 and WO2014/053622 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.

[0022] JACS, Vol 136, 2014, GJ Hilinski *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.

[0023] 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, peptide nucleic 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 5 or 4) as carriers.

[0024] 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[2,4,12-14].

[0025] However, their ability to penetrate cells and access their targets is compromised due to their uncharged nature[15].

[0026] Overcoming the problem of facilitating the entry of ON into cells is therefore desirable.

[0027] Other examples of drugs or BACs which are delivered in a targeted manner are DNA molecules (including linear and circularised molecules), RNA molecules and peptides.

[0028] Such strategies, including protein augmentation and viral mediated gene augmentation strategies have been the cornerstone of many medicines developments, however targeting issues and efficiencies of cargo delivery can constitute considerable problems.

[0029] Again, overcoming the problem of facilitating the entry of such DNA, RNA and peptides based BACs into cells is also desirable, *e.g.* circularised viral genomes in the absence of being packaged into infectious virions.

[0030] 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.

[0031] 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.

[0032] 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[16].

[0033] 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 of a BAC, such as an ON, per se.

[0034] 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.

[0035] 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).

[0036] However, since their first description[17] 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/][18], 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[19] and in higher mammals this manifests as prohibitive toxic side effects, such as tubular degeneration of the kidney[20].

[0037] 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.

[0038] 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[21]. In PCT/GB2016/054028, the applicant provided evidence that a reduction in formal charge of an 8 amino acid peptide from +3, to +2, +1 and 0 had interesting properties such that reduced charge variants retained cell entry properties. The applicant now bring forward data that reduced charged variants of CPP that have been cyclised by a ring closing metathesis have improved toxicological profiles when compared to the original CPP with a formal charge of +3.

[0039] 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. The applicant overcame this problem, as disclosed in PCT/GB2016/054028, and the present application is an extension thereof, which utilizes additional chemistries providing alternative stabilized peptides.

[0040] Previously, the applicant has 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.

[0041] PCT/GB2016/054028 taught preparing stapled and stitched peptides, two linked amino acids (stapled) or three or more linked amino acids (stitched), by incorporating 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**).

[0042] In addition, it is envisioned that ON's can be designed to hybridise to a single stranded or double stranded DNA or RNA molecules (or analogues thereof) such that the hybridised DNA or RNA molecule can be delivered to a target cell. These DNA and RNA may be linear, branched, circularised or adopt any stable conformation. These DNA and RNA may be synthetic, engineered or natural molecules, *e.g.* circular DNA molecules generated during viral genome replication that creates an episome.

[0043] [22,23]Stabilising peptides can be performed by crosslinking amino acids incorporated into defined relative position in the peptide sequence during solid phase synthesis or by chemically modifying existing amino acids. Cross-linking two specific amino acids (stapled) or cross-linking 3 or more amino acids (stitching) can be used to stabilize peptides of varying lengths. The specifically incorporated amino acids usually contain orthogonal functional groups allowing for specific and efficient cross-linking reactions to occur on resin or in solution post cleavage of the peptide. An examples of a cross-linking reaction based on resin ring closing metathesis of olefin baring amino acids is disclosed in PCT/GB2016/054028.

[0044] The present invention introduces additional CPPs based on alternative stapling or stitching technologies which introduce a cross link or bridge which provides a cyclisation between at least two amino acids. These include, but are not limited to:

a) the incorporation or derivatization of amino acids with functional groups for Huisgen 1,3 dipolar cyclo-additions, typically azidolysine and propargyl functional groups, such as, α-propargylalanine. Typically these reactions can be cyclized with a copper or a ruthenium based catalyst to yield 5 membered heterocyles, such as 1,2,3 triazoles with either 1,4 or 1,5 substitution favoured respectively. In addition to azide and propargyl functional groups, other substrates such as electron deficient nitriles and or diazoalkanes could be used[24]; and

b) the use of proteogenic amino acids for lactam formation by, for example, cyclizing lysine with a glutamic or aspartic acid residue within the peptide taking advantage of careful selection of protection groups during solid phase synthesis[25].

[0045] For persons skilled in the art, stapled CPPs can be developed based upon other cyclisation technologies. Combinations of stapled cyclisation based on a single methodology or several different crosslinking cyclisation methodologies could be utilized to form CPPs with either a single or multiple staples, either non-contiguously or contiguously distributed along a peptide. Further, persons skilled in the art can form StiP's that are similarly composed of one or more cyclisation technologies.

[0046] The cellular entry dynamics of existing linear CPPs 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[22,26]. Given that StiP's and StaP's uptake is abrogated with reduced cellular decoration of heparin sulphate[22] a macropinocytotic entry mechanism is inferred[27], suggesting this altered entry mechanism enables enhanced cellular uptake and bio-distribution compared to the state of the art.

[0047] 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[28]. It is likely that this increase in drug-likeness stems from the stabilised 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.

[0048] The importance of the stabilised CPP having a conformation imposed upon it should not be overlooked as the conformation imposed is responsible, in part, for the increased propensity for cellular uptake. The potential reduced or lack of membrane toxicity and immunogenicity enhances the clinical translatability of these compounds when conjugated to drugs and BAC's such as ON's.

[0049] The BAC and CPP can be covalently conjugated directly, or covalently conjugated via a BFL. Many functional groups may be used for conjugation reactions.

[0050] 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. Further, ONs can also be used to hybridise to a DNA or RNA molecule (or analogue), such as an episome, to facilitate its delivery, particular when conjugated to a StaP or StiP CPP.

[0051] As an 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. AOs can be designed to target 5' translation initiation start sites of viral gene transcript(s) to prevent binding of the translational machinery. Using AO to suppress viral translation is a well-established technology[3] and has progressed into clinical trials for viral haemorrhagic fevers such as Marburg and Ebola[4,5]. One PMO, AVI-7537 was evaluated for human use in the West African Ebola outbreak in 2014-15.

[0052] The use AOs have been adopted for other RNA steric block strategies. AOs can alter RNA splicing to either exclude an exon from the final processed mRNA, with Duchenne muscular dystrophy being the lead indication[2], or include an exon from the final processed mRNA, with Spinal Muscular Atrophy being the lead indication[1]. Whilst both AO led to successful FDA approvals in 2016, systemic delivery of the respective AO's remains a considerable hurdle.

[0053] Some tissues are particularly refractory to naked PMO transfection, *e.g.* heart, which may reflect differential vesicle-mediated PMO uptake mechanisms[26]. In fact, direct intra-cardiac injection of naked PMO does not even lead to efficient transfection[29], and refractory tissues tend to require repeat administration or high dose strategies[30-32]. However, whilst CPP conjugation improves PMO bio-distribution and serum stability[33-35], the toxicity associated with these linear, arginine rich peptides is still a major roadblock for pipeline development[20].

[0054] WO2016/187425 discloses an AO conjugated to a peptide that has been solely subject to cysteine arylation to form a bridge, no other technologies are disclosed. The cysteine arylation bridging technology is not a cyclisation technology, as it introduces rigid aromatic rings as the bridging moieties, thus it is not expected to have a stabilised conformation imposed upon it. Aside, the structure would not be stable following systemic administration due to the reduction of the thiol bonds which would release the bridge. In addition WO2016/187425 discloses arginine rich peptides only, therefore considerable membrane toxicity concerns remain for this technology.

[0055] Nitrogen arylation has been developed as a cyclisation technology[36]. Although it is possible to conjugate such peptides to a BAC, these peptides would not be expected to form stabilized structure and importantly do no enter cells better than traditional linear peptides[36].

[0056] For effective clinical translation of steric blocking AOs, CPPs need to effectively deliver the BAC to either the cytoplasm or nucleoplasm whilst limiting any toxicity associated with cell entry.

[0057] Thus, providing DCCPM's or DTCPM'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

[0058] 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),

the CPA is a stabilized peptide (CPP) which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP), the StiP or StaP comprises a cross link or bridge between at least two amino

acids of the peptide and the cross link or bridge provides a cyclisation between at least two amino acids which are not formed by an olefin metathesis.

[0059] Cyclisation may be achieved by one or more of:

i. condensation of an aldehyde or ketone with a hydrazine or protected hydrazine;
ii. a thiol-ene Michael addition;
iii. a di-sulfide formation;
iv. a Huisgen 1, 3 di-polar cycloaddition;
v. a reaction between an amine and carboxylic acid;
vi. a singlet or triplet based carbine reaction; or
vii. a Suzuki or Sonogashira coupling.

[0060] Particularly preferred cyclisations are obtained from chemistries iv) and v):

[0061] Using iv) a 5 membered heterocycle is formed between an azide or electron deficient nitrile containing amino acid and a propygyl containing amino acid.

[0062] Using v) a lactam is formed between a free amine containing amino acid and a carboxylic acid containing amino acid

[0063] A StaP may be formed by, for example, stapling two conformationally adjacent amino acids together, and a StiP may be formed by, for example, stitching at least three conformationally adjacent amino acids.

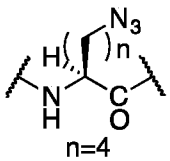
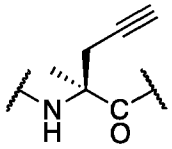
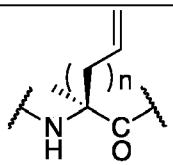
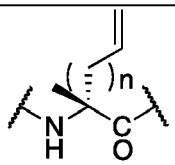
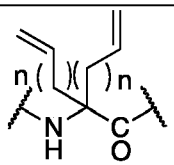
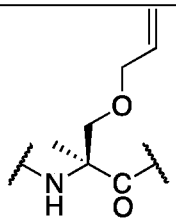
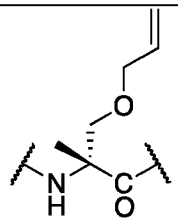
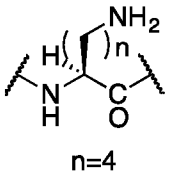
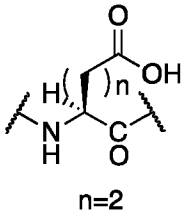
[0064] The stapling or stitching results in the formation of a cross link or bridge providing a cyclisation between two conformationally adjacent amino acids of the peptide.

[0065] In PCT/GB2016/054028 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.

[0066] The present invention discloses a cross link or bridge comprised of two orthogonal functional groups able to from covalent bond or bonds between the two functional groups as illustrated in Fig 1 (stapled peptides) and Fig 2 (stitched peptides).

[0067] The CPP preferably comprises at least two of the following: un-natural amino acids, proteogenic amino acids or modified protoegenic amino acids bearing functional groups as illustrated in Table 1 below.

[0068] Table 1

Unnatural or Modified Amino Acids				
 n=4		 n=6 n=2	 n=6 n=2	 n=6 n=2
				
Proteogent Amino Acids				
 n=4	 n=2 n=1			

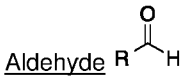
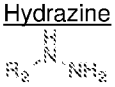
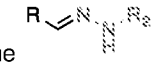
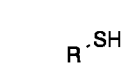
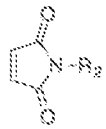
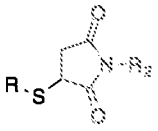
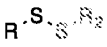
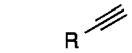
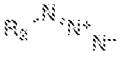
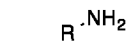
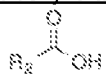
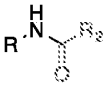
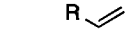
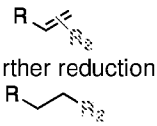
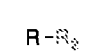
[0069] The preferred stapled or stitched CPPs incorporate one or more of the functional groups defined in Table 1, with an incorporated amino acid structure as shown in Fig 3. In Figs 1, 2 and 3, X or X_1 illustrate a functional group, such as those in Table 1. R can be hydrogen or a moiety such as methyl or isobutyl designed to sterically constrain the geometry of groups from the α-carbon, or R can = X or X_1 as defined in Table 1.

[0070] In PCT/GB2016/054028 the CPP preferably comprises at least two un-natural amino acids bearing all-hydrocarbon tethers (*e.g.* α-methyl,α-pentenyl glycine) and 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**).

[0071] Alternative CPPs and their method of manufacture are disclosed in Chu *et al*, 2014 and associated supplementary information, and are incorporated by reference[22].

[0072] The exemplified stabilized peptide comprises two or more orthogonal functional groups highlighted in Table 2 below that are covalently bound by the corresponding chemistry.

[0073] Table 2

Entry	Functional group (X)	Reacting Functional Group (X^1)	Resulting Functional Group
1	Aldehyde 	Hydrazine 	Hydrazone 
2	Thiol 	Maleimides 	Thioether 
3	Thiol 	Thiol 	Disulfide 
4	Alkyne 	Azide 	Triazole 
5	Amine 	Carboxylic acid 	Amide 
6	Diazirine 	Any	Various 
7	Olefin 	Olefin 	Olefin 
8	Aryl or Olefin R-M	Halide 	

[0074] The stabilized conformation typically comprises at least one alpha helix, extended 3_{10}-helix or poly (Pro) II 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 hairpin, or a combination of one or more of: an alpha helix, extended 3_{10}-helix or poly (Pro) II helix, turn, beta sheet or hairpin.

[0075] 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 below.

[0076] Table 3

Name	3-Letter Symbol	1-Letter Symbol	Molecular weight	Molecular formula	Residue formula	Residue Weight ($-H_2O$)	pK_a [a]	pK_b [b]	pK_x [c]	pI [d]
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	The	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

[a] pK_a is the negative of the logarithm of the dissociation constant for the $-COOH$ group
[b] pK_b is the negative of the logarithm of the dissociation constant for the $-NH_3^+$ group
[c] pK_x is the negative of the logarithm of the dissociation constant for any other group in the molecule
[d] pI is the pH at the isoelectric point

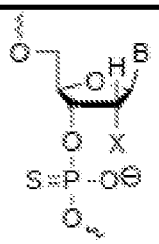
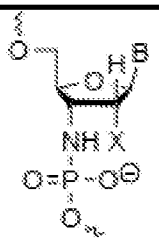
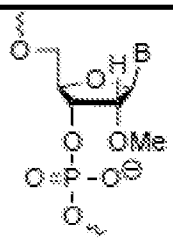
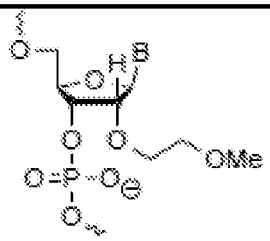
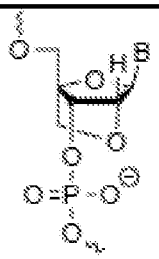
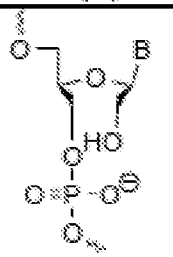
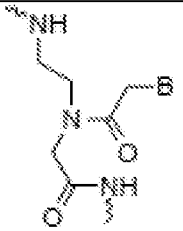
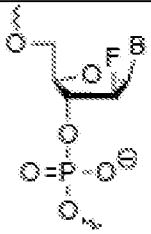
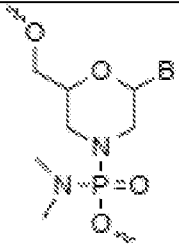
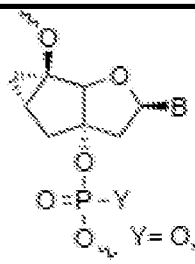
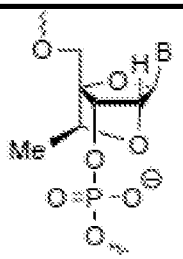
References: D. R. Lide, *Handbook of Chemistry and Physics, 72nd Edition*, CRC Press, Boca Raton, FL, 1991.

[0077] 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.

[0078] Accordingly, it is possible to reduce the charge on the peptide sequences.

[0079] The preferred BAC is an oligonucleotide (ON), more preferably still an anti-sense oligonucleotide (AO). Different anti-sense oligonucleotide chemistries are illustrated in Table 4 below, with the use of low charge or neutral charged chemistries, such as, phosphorodiamidate morpholino oligonucleotides (PMOs) being preferred.

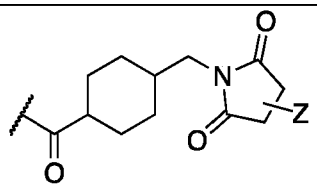
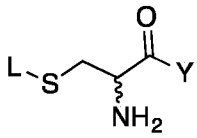
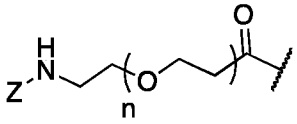
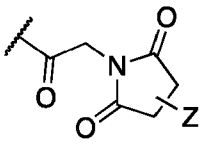
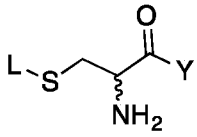
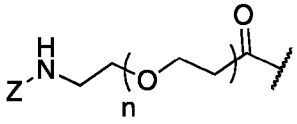
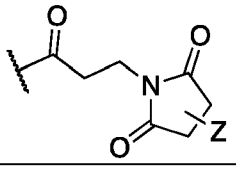
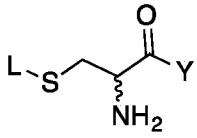
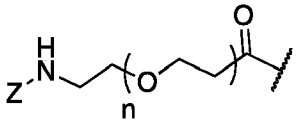
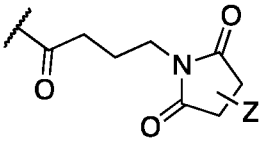
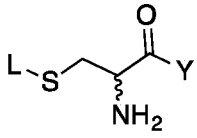
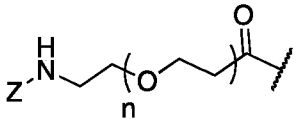
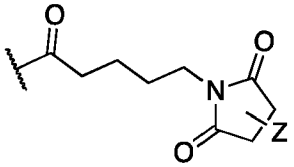
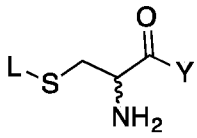
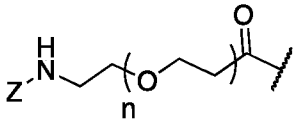
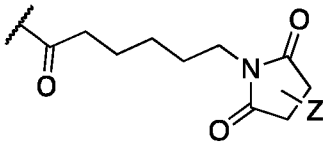
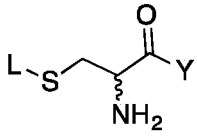
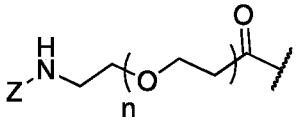
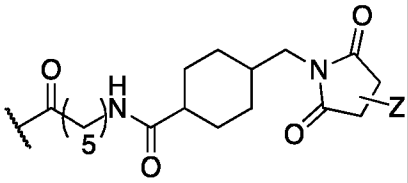
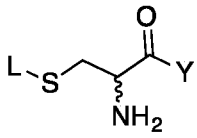
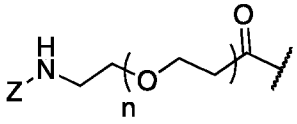
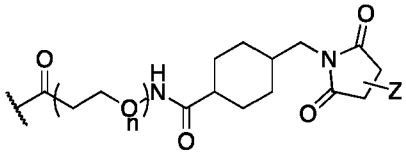
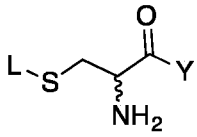
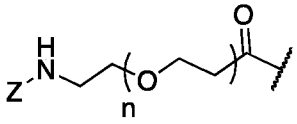
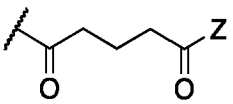
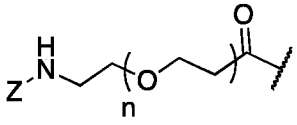
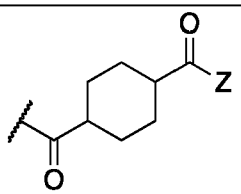
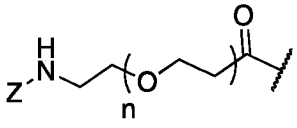
[0080] Table 4

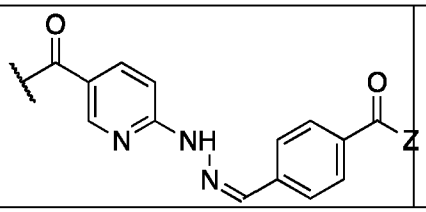
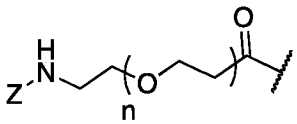
 Phosphorothioate (**PS**)	 N'3 Phosphoramidite (**NP**)	 2'-O-Methyl (**2' O-Me**)
 2'-O-Methoxyethyl (**MOE**)	 Locked Nucleic Acid (**LNA**)	 Unlocked Nucleic Acid (**UNA**)
 Peptide Nucleic Acid (**PNA**)	 2'F-Arabino Nucleic Acid (**2'F-ANA**)	 Phoshoramidate morpholino (**PMO**)
 Tricyclo-DNA (**tcDNA**)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

[0081] 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.

[0082] Whilst the BAC may be linked to the CPP directly (Fig 4) the applicant has found the use of a BFL desirable. Exemplary, non-limiting BFL chemistries are illustrated in Table 5 below.

[0083] 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		HYNIC	Not present	 or not present

[0084] By way of a footnote to Table 5, the following should be noted:

[0085] Fig 5a and 5b and Fig 5c and 5d highlights general structure of a DCCPM where the following are preferred, but not limited to the following defined atoms or groups.

[0086] In a preferred embodiment illustrated in Fig 5a, 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 (Fig 5b).

[0087] Other embodiments may utilize variations over the structure shown in Fig 5b. For example if another embodiment does not require a thiol for conjugation of the BFL to the CPA as illustrated in Fig 5c, then Z = Y3, where Y3 is a spacer in Table 5. For a BFL that does not require a sulfur 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.

[0088] 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 Fig 4, then the following apply. The resin utilized in the solid phase synthesis of the peptide would be changed from rink amide or rink amide MBHA to 2-chlorotrityl resin to yield a peptide with a free carboxylic acid able to undergo coupling via standard peptide coupling conditions.

[0089] The orthogonal functional groups highlighted in Table 1 can also be used in a bio-conjugation reaction. These functional groups can be used to conjugate molecules to the DCCPM 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 phospholipid, a polyphenol, a nuclear localising signal, a nuclear export signal, an antibody, and a targeting molecule.

[0090] A preferred linker chemistry utilises an amine to sulfhydryl 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 and the BFL and BAC illustrated in Fig 7a-d.

[0091] 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.

[0092] In the preferred embodiment of any of the above CPAs *e.g.* (Fig 7a), may be covalently linked to a BFL, if required, preferentially incorporating $(PEG)_n$ where n=1-10 linked to the N terminus of the peptide and terminated with a sulphur containing molecule *e.g.* cysteine (Fig 7b). This allows further extension of the BFL with a bi-functional reactive molecule such as (SMCC)

(Fig 7c). This in turn is covalently linked to a functional group on the BAC, in the preferred embodiment a primary amine, thus generating a DCCPM (Fig 7d).

[0093] Covalent linkage to the CPP may be via, for example, but not limited to, a β-ala or for someone skilled in the art any other suitable moiety which may include a branched or dendrimer like structure allowing for multiple BAC or CPPs to be conjugated.

[0094] In any particular embodiment the relative position of the cross-linking amino acids are referred to by their positioning within the sequence where the C terminus is the first amino acid referred to as positon 1 and subsequent amino acids are number in a N to C terminus fashion. A descriptor of the functionality is defined in Table 2. For example, a cross-link between a lysine reside (K) and glutamic acid reside (E) in the sequence RKF-[E-RLF-K] hitherto will be termed K1E5-8M (8M refers to an 8 mer amino acid), brackets in the sequence represents the cyclic portion of the peptide. Similarly for the sequence RKF-[S5-RLF-S5] cross linked between the S5 monomer by a ring closing metathesis will be referred to RCM1,5-8M.

[0095] In any particular embodiment the sequence of RKF-E-RLF-K will be a sequence of K1E5-8M not cyclized between the lysine and glutamic residue, hitherto referred to as K1E5-8M-NC.

[0096] In any particular embodiment where the relative spacing between the crosslinked amino acids is decreased, such as the sequence RKFR-[E-LF-K], will therefore be referred to as K1E4-8M; or relative spacing between the crosslinked amino acids is increased such RK-[E-FRLF-K] will be referred to as K1E6-8M.

[0097] Hereafter, where the CPP comprises the sequence RKF-[E-RLF-K] and the BFL is a PEGylated SMCC, the resultant compound is termed K1E5-CP8M (Fig 7c).

[0098] 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 to a cell penetrating agent (CPA) which is a stabilized peptide that has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP), the StiP or StaP comprises a cross link or bridge between at least two amino acids of the peptide and the cross link or bridge provides a cyclisation between the at least two amino acids which are not formed by an olefin metathesis, 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.

[0099] In another embodiment of any of the above CPAs *e.g.* (Fig 8a), $(PEG)_n$ is linked to the N terminus of the peptide and terminated with a hydrazynal nicotinic acid (HNA) (Fig 8b). This allows conjugation of BFL with the appropriately modified BAC, in this instance the BAC is terminated with an aromatic aldehyde preferentially 4-formyl benzoic acid (Fig 8c). This in turn is covalently linked to a functional group on the BAC to form a DCCPM (Fig 8d).

[00100] Where the CPP comprises the sequence RKF-[E-RLF-K] and the BFL is a PEGylated hydrazynal nicotinic acid (HNA), the resultant compound is termed K1E5-HP8M (Fig 8b).

[00101] Where the CPP comprises the sequence RKF-[E-RLF-K] and the BFL is SMCC with no PEGylation incorporated, the resultant compound is termed K1E5-C8M (Fig 6d).

[00102] In any particular embodiment, a CPP derived from cyclizing lysine with a glutamic or aspartic acid residue can be directly conjugated to a BAC, such as an AO (Fig 9).

[00103] In any particular embodiment, the method of forming a crosslink between two amino acid, modified amino acids or un-natural amino acids can be any and not limited to functional groups defined in Table 1.

[00104] In another preferred embodiment the orthogonal functional groups used for forming a cross-link are an incorporated azide residue and an alkyne residue.

[00105] Specifically in a cross link that used an azide and alkyne, the residues incorporated are (S)-N-Fmoc-2-(2'-propynyl)alanine baring an alkyne and (2S)-N-Fmoc-6-azido-hexanoic acid baring an azide.

[00106] In the event of incompatible synthesis or unfavorable cleavage condition of modified amino acids, some residues can be converted *in situ*, *e.g.* the treatment of lysine with N-diazo-1,1,1-trifluoromethanesulfonamide (TfN_3) to yield the azide functional group or the use of a di-azide molecule such as 1,3-benzenedicarbonyl di-azide or similar substituted di-azide to bridge between two propargyl containing amino acids.

[00107] In any particular embodiment the relative position of the cross linking amino acids are referred to by their positioning within the sequence and a descriptor of the functionality used and defined in table 1. Such as a cross-link between a (2S)-N-Fmoc-6-azido-hexanoic acid abbreviated to K(N_3) and (S)-N-Fmoc-2-(2'-propynyl)alanine abbreviated to B in the sequence RKF-[K(N_3)-RLF-B] will be referred to as B1K(N_3)5-8M (brackets in the sequence represents the cyclic portion of the peptide).

[00108] In any particular embodiment the sequence of RKF-K(N_3)-RLF-B will be a sequence of B1K(N_3)5-8M not cyclized between the azidolysine and propylgyl residue, will be hitherto referred to as B1K(N_3)5-8M-NC.

[00109] In any particular embodiment where the relative spacing between the amino acids is altered, this will be referred to as B1K(N_3)4-8M in case of a decrease (such as the sequence RKFR-[K(N_3)-LF-B]), or as B1(KN_3)6-8M in case of an increase (such as RK-[K(N_3)-FRLF-B]).

[00110] Hereafter, where the CPP comprises the sequence RKF-[K(N_3)-RLF-B] and the BFL is a PEGylated SMCC, the resultant compound is termed B1K(N_3)5-CP8M (Fig 10c).

[00111] Where the CPP comprises the sequence RKF-[K(N_3)-RLF-B] and the BFL is a PEGylated hydrazynal nicotinic acid (HNA), the resultant compound is termed B1(KN_3)5-HP8M (Fig 11b).

[00112] Where the CPP comprises the sequence RKF-[K(N_3)-RLF-B] and the BFL is SMCC with no PEGylation incorporated, the resultant compound is termed B1K(N_3)5-C8M.

[00113] As highlighted in Fig 5d, a StaP or StiP conjugated ON (either with or without a BFL) can constitute a delivery vehicle for other BAC's. The ON could hybridise or associate with BAC's including DNA, RNA or protein molecules. The resulting molecules are referred to as Drug Targeting Cell Penetrating Molecules (DTCPM's). Of note, DTCPMs can hybridise to circular DNA or RNA molecules (episomes) or linear DNA or RNA molecules that are naturally generated during viral genome replication; in particular when viral packaging signals are not provided such that viral genomes are not packaged to form mature infectious virus particles.

[00114] According to a third aspect of the present invention there is provided a DCCPM or a DTCPM 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.

[00115] The DCCPM may be used in the treatment of, for example, a neuromuscular disease, metabolic disease, cancer, age-related degenerative disease or to treat an acquired infection.

[00116] The DTCPM may be used in the treatment of, for example, a neuromuscular disease, metabolic disease, cancer, age-related degenerative disease or to treat an acquired infection.

[00117] 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.

[00118] In the case of DMD the DCCPM may comprise an AON targeting exon 51 of the dystrophin gene.

[00119] 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) and the StiP or StaP comprises a cross link or bridge between at least two amino acids of the peptide and the cross link or bridge provides a cyclisation between the at least two amino acids which are not formed by an olefin metathesis.

[00120] The shape imposed by the stabilization may generate a structure with a minimized free energy conformation.

[00121] 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). The StiP or StaP comprises a cross link or bridge between at least two

amino acids of the peptide and the cross link or bridge provides a cyclisation not formed by an olefin metathesis and administering it to a subject.

[00122] 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.

[00123] 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 or DTCPM of the invention to a subject.

[00124] In accordance with a seventh aspect of the present invention there is provided a composition comprising a DCCPM or DTCPM 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

[00125] Embodiments of the invention are further described hereinafter with reference to the accompanying drawings, in which:

[00126] Figs 1a-c illustrate examples of un-cyclised CPPs (left) and some of the resulting stabilized structures (right) that can be formed therefrom;

[00127] Figs 2a-d illustrate examples of stitched CPA's: Fig 2a is formed by cyclizing two sets of orthogonal amino acid that are consecutively placed in the peptide sequence; Fig 2b illustrates stitched CPA's formed by cyclizing two different sets of orthogonal amino acids not consecutively placed in the peptide sequence; Fig 2c illustrates a stitched CPA where two sets of amino acids to be cross-linked originate from one amino acid; and Fig 2d illustrates two alternative sets of amino acids that originate form one amino acid that can be cross linked to form a continuous stitched CPA;

[00128] Fig 3 exemplifies the structure of an amino acid that can be incorporated into CPPs with varying functional groups as defined in Table 1. The functional groups presented by X and X_1 can be cross-linked to form CPAs and can also be used for bio-conjugation;

[00129] Fig 4 illustrates the direct conjugation of a stabilized CPP to a BAC without the use of BFL;

[00130] Fig 5 illustrates the connectivity of BFLs and overall structure of DCCPM and DTCPM.

[00131] Figs 5a is a schematic diagram of a DCCPM illustrating the connectivity of BFL with non-limiting examples from Table 5 where the BAC is an antisense oligonucleotide (AO) or an oligonucleotide (ON);

[00132] Figs 5b-c are schematic diagrams of a DCCPM illustrating the connectivity of BFL with non-limiting examples from Table 5 where the BAC is an AO or an ON where the CPA has

been stabilized by a either a 1,4 substituted or a 1,5 substituted 1,2,3 triazole;

[00133] Fig 5d is a schematic diagram of a DTCPM illustrating the connectivity of BFL with non-limiting examples from Table 5 where oligonucleotide constitute part of the delivery molecule; a non-limiting list of BAC's delivered by this technology includes DNA, RNA or protein molecules (in effect the ON is part of the BFL and connects to the BAC);

[00134] Fig 6 illustrates some preferred embodiments of DCCPMs incorporating different BFLs.

[00135] Fig 6a is a general schematic of a DCCPM and illustrates connectivity of BFL defined but not limited to Table 5;

[00136] Fig 6b is a schematic diagram illustrating a preferred embodiment of an DCCPM with an lactam stabilized CPP with an incorporated PEG_5 which is terminated with a thiol containing molecule (cysteine) and conjugated to the BAC using the heterobifunctional protein cross linker Succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC);

[00137] Fig 6c is a schematic diagram illustrating a preferred embodiment of a DCCPM with an lactam stabilized CPP with an incorporated PEG_5 which is terminated with a hydryzynal nicotinic acid (HNA) and subsequently conjugated to a BAC which has been modified with 4-formyl benzoic acid;

[00138] Fig 6d is a schematic diagram illustrating a preferred embodiment of an DCCPM with an lactam stabilized CPP which is terminated with a thiol containing molecule (cysteine) and conjugated to the BAC using the heterobifunctional protein cross linker Succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC);

[00139] Figs 7 illustrates some preferred embodiments of DCCPMs incorporating CPPs stabilized by crosslinking lysine and glutamic residues forming a lactam;

[00140] Fig 7a is a schematic of a CPP stabilized by cyclisation between two proteogenic amino acids to form a lactam;

[00141] Fig 7b illustrates the incorporation of PEG_5 to the N terminus of the CPA and its termination with a thiol containing molecule (cysteine);

[00142] Fig 7c illustrated the incorporation of the hetero bi-functional linker SMCC into the DCCPM;

[00143] Fig 7d is a schematic diagram illustrating a preferred embodiment of a DCCPM with an K-E stabilized CPP with an incorporated PEG_5 which is terminated with a thiol containing molecule (cysteine) and subsequently conjugated to a BAC using the heterobifunctional protein cross linker Succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC);

[00144] Figs 8 illustrates some preferred embodiments of DCCPMs incorporating CPPs stabilized by crosslinking lysine and glutamic residues forming a lactam;

[00145] Fig 8a is a schematic of a CPP stabilized by cyclisation between two proteogenic amino acids to form a lactam;

[00146] Fig 8b is a schematic diagram illustrating a preferred embodiment of an DCCPM with an lactam stabilized CPP with an incorporated PEG_5 which is terminated with a hydrazynal nicotinic acid (HNA) and subsequently conjugated to a 4-formyl benzoic modified BAC;

[00147] Fig 8c a schematic diagram of a 4-formyl benzoic modified BAC;

[00148] Fig 8d is a schematic diagram illustrating a preferred embodiment of a DCCPM with an lactam stabilized CPP with an incorporated PEG_5 which is terminated with a hydryzynal nicotinic acid (HNA) and subsequently conjugated to a BAC which has been modified with 4-formyl benzoic acid;

[00149] Fig 9 is a schematic diagram illustrating a preferred embodiment of a directly conjugated lactam containing CPP to a BAC;

[00150] Figs 10 are schematic diagrams illustrating the conjugation of a 1,4 substituted triazole stabilised CPP to a BAC to form a preferred embodiment of a DCCPM;

[00151] Fig 10a is a schematic diagram of a 1,4 substituted triazole stabilised CPP;

[00152] Fig 10b is a schematic diagram of a 1,4 substituted triazole stabilised CPP with an incorporated PEG_5 and terminated with a thiol-containing molecule (cysteine);

[00153] Fig 10c is a schematic diagram of a 1,4 substituted triazole stabilised CPP and illustrates the incorporation of the heterobifunctional protein cross linker Succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC);

[00154] Fig10d is a schematic diagram illustrating a 1,4 substituted triazole stabilized CPP as part of a DCCPM. It should be noted that the 1,2,3 triazole could contain other isomers *e.g.* 1,5 substituted triazole;

[00155] Fig 11a illustrates connectivity of 1,4 substituted triazole containing CPP;

[00156] Fig 11b is a schematic diagram illustrating a preferred embodiment of a DCCPM with a triazole stabilized CPP with an incorporated PEG_5 which is terminated with a hydrazynal nicotinic acid (HNA) and subsequently conjugated to a 4-forml benzoic modified BAC;

[00157] Fig 11c a schematic diagram of a 4-formyl benzoic modified BAC;

[00158] Fig 11d is a schematic diagram illustrating a preferred embodiment of a DCCPM with a 1,4 substituted triazole stabilized CPP with an incorporated PEG_5 which is terminated with a hydryzynal nicotinic acid (HNA) and subsequently conjugated to a BAC which has been modified with 4-formyl benzoic acid;

[00159] Fig 12 is circular dichroism spectra of K1E4-CP8M, K1E5-CP8M and K1E6-CP8M blanked to D2O and collected at room temperature and in triplicate. K1E5 displays a random coil, where as K1E4 and K1E6 show extended Pi helices;

[00160] Fig 13 is flow cytometry data for HeLa pLuc 705 cells incubated in the presence of FITC labeled CPAs;

[00161] Fig 13a(i) is a comparison of FL1+ cells of HeLa pLuc 705 cells incubated with K1E4-P8M-FITC or K1E4-P8M-FITC-NC 4 hours n = 4, error bars show standard deviation 2 way

ANOVA was performed and showed no interaction affect. Data illustrates that there was no significant difference in the number of FL1 positive cells when K1E4-P8M-FITC was cyclized;

[00162] Fig 13a(ii) is a comparison of FL1+ cells of HeLa pLuc 705 cells incubated with K1E5-P8M-FITC or K1E5-P8M-FITC-NC 4 hours n = 4, error bars show standard deviation 2 way ANOVA was performed and showed no interaction affect. Data illustrates that there was no significant difference in the number of FL1 positive cells when K1E5-P8M-FITC was cyclized;

[00163] Fig13a(iii) is a comparison of FL1+ cells of HeLa pLuc 705 cells incubated with K1E6-P8M-FITC or K1E6-P8M-FITC-NC for 4 hours. n = 4, error bars show standard deviation 2 way ANOVA was performed * represents $p<0.05$, ** represents $p<0.001$. Data illustrates that there is a significant increase in the number of FL1 positive cells at a given concentration when K1E6-P8M-FITC is cyclized;

[00164] Fig 13b(i) is a comparison of MFI of HeLa pLuc 705 cells incubated with K1E4-P8M-FITC or K1E4-P8M-FITC-NC for 4 hours. n = 4, error bars show standard deviation. 2 way ANOVA was performed and showed no interaction affect;

[00165] Fig 13b(ii) is a comparison of MFI of HeLa pLuc 705 cells incubated with K1E5-P8M-FITC or K1E5-P8M-FITC-NC for 4 hours. n = 4, error bars show standard deviation. 2 way ANOVA was performed and showed no interaction affect;

[00166] Fig 13b(iii) is a comparison of MFI of HeLa pLuc 705 cells incubated with K1E6-P8M-FITC or K1E6-P8M-FITC-NC for 4 hours. n = 4, error bars show standard deviation. 2 way ANOVA was performed * represents $p<0.05$;

[00167] Fig 13c is a comparison % viable cells judged by gated cells compared to negative controls of HeLa pLuc 705 cells incubated with (i) K1E4-P8M-FITC versus K1E4-P8M-FITC-NC, (ii) K1E5-P8M-FITC versus K1E5-P8M-FITC-NC and (iii) K1E6-P8M-FITC versus K1E4-P8M-FITC-NC for 4 hours. n = 4, error bars show standard deviation. Data shows that there is no significant difference in the % of cells recovered when normalized to untreated controls;

[00168] Figs 14a-c are flow cytometry data for HeLa pLuc 705 cells incubated in the presence of FITC labeled CPAs;

[00169] Fig 14a is a comparison of FL1+ cells of HeLa pLuc 705 cells incubated with RCM1,5-P8M-FITC-3+, RCM1,5-P8M-FITC-2+, RCM1,5-P8M-FITC-1+, RCM1,5-P8M-FITC-0+, for 4 hours. n = 5, error bars show standard deviation. * represents $p<0.05$, ** represents $p<0.001$. *a represents an interaction between RCM1,5-P8M-FITC-1+ and all other groups, *b represents an interaction between RCM1,5-P8M-FITC-0+ and all other groups. Data shows that RCM1,5-P8M-FITC-1+ has a significant reduction in FL1+ cells compared to other treatments over a range of concentrations. RCM1,5-P8M-FITC-0+ shows in increase in the number of FL1+ cells over a range of concentrations compared to other treatments;

[00170] Fig 14b is a comparison of MFI of HeLa pLuc 705 cells incubated with RCM1,5-P8M-FITC-3+, RCM1,5-P8M-FITC-2+, RCM1,5-P8M-FITC-1+, RCM1,5-P8M-FITC-0+ for 4

hours. n = 5, error bars show standard deviation. * represents p<0.05, ** represents p<0.001. There is no significant difference in the MFI between RCM,1,5-P8M-FITC-0+, 1+ and 2+ at a given concentration. However RCM1,5-P8M-FITC-3+ shows an increase in MFI at higher concentrations > 1 μM;

[00171] Fig 14c is a comparison of % viable cells judged by gated cells compared to negative controls of HeLa pLuc 705 cells incubated with RCM1,5-P8M-FITC-3+ [RKF(**S5**RLF**S5**)], RCM1,5-P8M-FITC-2+ [RIF(**S5**RLF**S5**)], RCM1,5-P8M-FITC-1+ [IIF(**S5**RLF**S5**)], RCM1,5-P8M-FITC-0+ [IIF(**S5**ILF**S5**)] for 4 hours. n = 3, error bars show standard deviation * represents p<0.05. *a represents an difference between RCM1,5-P8M-FITC-3+ and RCM1,5-P8M-FITC-2+, *b represents an difference between RCM1,5-P8M-FITC-2+ and RCM1,5-P8M-FITC-0+ and *c represents an difference between RCM1,5-P8M-FITC-0+ and RCM1,5-P8M-FITC-3+. Data shows that at highest concentration 100 μM a significant loss in the number of viable cells recovered when comparing RCM1,5-CP8M-FITC-3+ to RCM1,5-CP8M-FITC-2+ and an increase in the number of cells when comparing RCM1,5-CP8M-FITC-2+ to RCM1,5-CP8M-FITC-0+. Similarly at 33 μM RCM1,5-CP8M-FITC-3+ compared to RCM1,5-CP8M-FITC-2+ and RCM1,5-CP8M-FITC-0+ showed a decrease in the % viable cells. At all other concentration no other significant affects were observed apart from at 0.05 μM. At 0.05 μM RCM1,5-CP8M-FITC-0+ showed an increase in viable cells over RCM1,5-CP8M-FITC-3+ and RCM1,5-CP8M-FITC-2;

[00172] Fig 15 illustrates % cell viability of HeLa pLuc 705 cells when comparing treatment with RCM1,5-P8M-3+-FITC to K1E5-P8M-FITC for 4 hours. n = 3-4, error bars show standard deviation * represents p<0.001. At all concentrations an increase in viability was observed when comparing K1E5-P8M-FITC to RCM1,5-P8M-FITC-3+.

DETAILED DESCRIPTION

[00173] The invention demonstrates that different stapling and stitching chemistries (to that disclosed in applicants earlier application PCT/GB2016/054028) can be used to produce drug carrying cell penetrating molecule (DCCPM) and that these stabilized peptides can confer different and potentially beneficial effects such as, for example, lower toxicity.

[00174] An exemplary drug carrying cell penetrating molecule (DCCPM) was produced with a FITC label in order to demonstrate cellular uptake.

[00175] The exemplary DCCPM comprises:

i) a biologically active compound (BAC);

ii) a cell penetrating agent (CPA) which is a stabilized peptide; and

iii) a bi-functional linker (BFL).

[00176] The three components forming the DCCPM are described in more detail below, and particularly favored embodiments are illustrated by way of reference to Figs 7a-d and Figs 8a-d.

1. **The Biologically Active Compound**.

[00177] 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 peptide nucleic acids (PNAs) or PMOs or their modified derivatives that might impart a small electric charge (either positive or negative).

[00178] 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.

[00179] 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.

[00180] 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 AO to suppress viral translation is a well-established technology and has progressed into clinical trials for viral haemorrhagic fevers such as Marburg and Ebola.

[00181] Also, ON can be designed to target 3' untranslated region of an endogenous transcript that alters the nuclear export, translation and stability of the transcript. Such targets include, but are not limited to polyadenylation and/or cleavage sites of the transcript.

[00182] 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.

[00183] Non-limiting exemplary ON chemistries are illustrated in Table 4.

[00184] In the non-limiting example illustrated, the target is exon 51 of the dystrophin gene and comprises the sequence:

Sequence id 1: 5'CUCCAACAUCAAGGAAGAUGGCAUUUCUAG3'

2. The Cell Penetrating Agent (CPA) which is a stabilized peptide

[00185] The cell penetrating agents of the invention are stabilized peptides.

[00186] The peptides may be stabilized by cross linking of 2 amino acids, modified amino acids or un-natural amino acids, to form a stapled peptide (StaP) or crosslinking 3 or more residues to form a stitched peptide (StiP).

[00187] Crosslinking by stapling and stitching may confer a property, *e.g.* a solvated conformation such as, but not limited to, an alpha helix, extended 3_{10}-helix or poly (Pro) II helix, a turn (for example, but not limited to, α, β, γ, δ or π), several turns to form a beta sheet or a hairpin, or a combination of one or more of: an alpha helix, extended 3_{10}-helix or poly (Pro) II helix, turn, or beta sheet, an energetic conformational bias dependent on solvation environment *e.g.* interaction with plasma membrane, cellular penetrance, and biological activity.

[00188] Non-limiting examples of alternative chemistries to that described in PCT/GB2016/054028 for producing StaP and StiP are illustrated in Table 6 and include peptide sequences with nominal position for cross linking by amino acid, modified amino acids or unnatural amino acids illustrated by X and refereeing to functional groups defined but not limited to Table 1.

[00189] Stabilisation of peptides *e.g.* stitching or stapling, can be performed by a variety of means dependent on the functional group incorporated into the peptide. Non-limiting examples of functional groups are demonstrated in Table 2. Some reactions require catalyst or have preferential reagents for stabilization and are illustrated in Table 6 below.

[00190] **Table 6**

Table 1 Entry	Brief description	Catalyst or desirable reagent
1	Condensation of aldehyde or ketone with hydrazine or protected hydrazine	Aniline as a catalyst
2	Thiol-ene Michael addition	Reducing agent such a immobilized TCEP or suitable base or nucleophile.
3	Di-sulfide formation	Reducing agent such as DTT or TCEP.
4	Huisgen 1,3 di-polar cycloaddition.	Typically Cu or Ru catalyzed reaction between azide and alkyene, but can include electron deficient nitriles.
5	Amide from corridponding amine and carboxylic acid	Standard peptide coupling conditions eg coupling reagent HATU, HOBt, COMU with base.
6	Singlet or triplet based carbene reaction	UV irridation at ≈355 nm
7	Olefin metathesis	Ruthenium based grubbs generation 1 and 2 and Grubbs-Hoyveda gen 1 and 2
8	Suziki or Sonogashira coupling	Palladium based catalysts

[00191] 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.

[00192] 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.

[00193] Non-limiting examples of unnatural amino acids used in stabilising a peptide structure are illustrated in Table 1.

[00194] In PCT/GB2016/054028 the applicant employed α,α-disubstituted unnatural amino acids bearing all-hydrocarbon tethers (*e.g.* α-methyl,α-pentenyl glycine).

[00195] In the present invention the applicant in the alternative employs the chemistries disclosed in Table 6.

[00196] In one preferred embodiment they form a cross link by coupling two naturally occurring amino acid (*e.g.* lysine and glutamic acid) in the sequence RKF-[E-RLF-K]. Alternatively, these naturally occurring amino acids could be lysine and aspartic acid.

[00197] In yet another embodiment the applicant employs a cross link between a modified natural amino acid (N^6-diazolysine) and a non-natural amino acid (S)-2-Amino-2-methyl-4-

pentynoic acid such as the sequence RKF-[K(N_3)-RLF-B] where B represents (S)-2-Amino-2-methyl-4-pentynoic acid and K(N_3) represent N^6-diazolysine.

[00198] In one embodiment the cell penetrating agent has a staple or stitch peptide comprising the sequence RFK-X-RLF-X, where X represents an amino acid that is able to be cross linked.

[00199] In another embodiment the sequence RFK-[X-RLF-X] could have the relative position of the cross linking residues moved, for example, but not limited to RF-[X-KRLF-X] or RFKR-[X-LF-X].

[00200] 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.

[00201] 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.

[00202] Peptide sequence can include modified amino acids (Table 1) 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 phospholipid, a polyphenol, a nuclear localising signal, a nuclear export signal, an antibody and a targeting molecule.

3. Bi-Functional Linker

[00203] A bi-functional linker may be used to link the BAC to the CPA.

[00204] 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.

[00205] 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.

[00206] 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 9 and 10).

[00207] 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. Non-limiting examples are shown in Table 5.

[00208] 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.

EXAMPLES

PROOF OF PRINCIPAL PROVIDED IN PCT/GB2016/054028 WITH PMO CP8M AND PMO HP8M WHICH SERVE AS COMPARATORS TO THE ALTERNATIVE CHEMISTRIES DESCRIBED HEREIN.

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

Introduction

[00209] 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[37]. 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[38,39].

[00210] RNA splicing suppression of the DMD transcript has particular promise. The hybridisation of AOs 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. AO-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[32,40-45], which led to clinical development programs[2,10].

[00211] Although intravenously administered PMOs have demonstrated a dose-dependent increase in dystrophin re-expression with some functional benefit[2,46], 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[30-32].

[00212] To date unmodified CPA conjugation improves PMO bio-distribution and serum stability[33-35], however toxicity is still a major roadblock for pipeline development[20].

[00213] The 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. We bring data forward that demonstrate novel CPA have attractive biological and toxicological properties, such that these novel DCCPMs or DTCPMs are clinically relevant.

Materials and Methods

General Peptide Synthesis Procedure

[00214] For ring closing metathesis peptides, 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.

[00215] All peptides were synthesized following an established protocol using standard Fmoc-peptide chemistry on Rink amide MBHA resin or 2-chlorotrytyl resin for the free acid variants. 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.

Synthesis of peptide containing modified amino acids and un-natural amino acids.

[00216] Deprotection of the temporary Fmoc group was achieved by 2 × 20 min treatments of the resin- bound peptide with 20% (v/v) piperidine in DMF. After extensive flow washing with DMF, coupling of each successive amino acid was achieved with 1 × 30 min incubation with the appropriate preactivated *N*R-Fmoc-amino acid derivative. All protected amino acids (1 mmol) were dissolved in the cartridge with 3.8 mL of 0.25 M DEPBT in DMF as part of the synthesizer program immediately before delivery to the reaction vessel. Subsequently, 1 mL of DIEA was added directly to the cartridge to effect activation no more than two minutes prior to transfer of the coupling solution to the NR-deprotected resin-bound peptide. After coupling was complete, the resin was extensively flow washed in preparation for the next deprotection/coupling cycle.

Synthesis for lactam containing amino acids (*e.g.* K1E5)

Side-chain protection of glutamic acid and lysine consisted of Fm and Fmoc groups, respectively. After acetylation amino terminus with Ac2O/NMM (2 mmol each, 114 and 189 µL, respectively) in a manner similar to any other *N*R-Boc-amino acid, Fmoc, and Fm side-chain protecting groups were removed with 20% piperidine, the resin-bound peptide was washed with DMF and then treated for 2 h with 1.9 mmol of HBTU (3.8 mL × 0.5 M in DMF) and 1.9 mmol of DIEA to effect the lactam formation.

Selective transformation of Lysine to N^6-diazolysine

[00217] The target lysine residue for transformation was protected with an Mtt protecting group where as other lysine residues that do not require transformation are protected with Boc protecting group. The resin-bound peptide (0.31 mmol/g, 300 mg) in this instance the sequence RK(Boc)F-K(Mtt)-RLF-B, where B is the incorporated non-natural amino acid (S)-N-Fmoc-2-(2'-propynyl)alanine was suspended in 1% TFA solution in DCM (10 mL) and was stirred for 3 min. The solution becomes yellow instantaneously. Then the resin was washed with DCM (2×), MeOH (1×), and DCM (2×). The process was repeated 8 times until the solution stayed colorless. The resin-bound peptide was taken to the next step without further manipulations. Triflic acid anhydride (Tf_2O, 316 µL, 1.87 mmol) was added dropwise to a vigorously stirred mixture of NaN3 (600 mg, 9.2 mmol) in H_2O (1.5 mL) and CH_2Cl_2 (3 mL) at 0 °C. The resulting mixture was allowed to warm to room temperature and was stirred for 2 h. The water layer was extracted twice with CH_2Cl_2, and the combined organic layers were washed with saturated aqueous Na_2CO_3. The resulting solution of TfN_3 in CH_2Cl_2 was added slowly to the resin-bound peptide suspended in a solution of $CuSO_4 \cdot 5H_2O$ (2 mg, 8 mmol) and K_2CO_3 (5 mg, 36 mmol) in MeOH (1 mL). This reaction mixture was swirled for 18 h at room temperature. The completeness of the diazo transfer could be followed with the Kaiser test; colorless resin beads implied that the conversion of the

amino group into the azido functionality had been completed. The resin was subsequently washed sequentially with DCM, MeOH, DMF, and DCM.

Cleavage and de protection of the peptide

[00218] The azide/alkynyl-containing resin-bound peptide was deprotected and cleaved from the solid support by treatment with TFA/TIS/H_2O (95:2.5:2.5 v/v) for 4h at RT. After filtration of the resin, the TFA solution was concentrated under reduced pressure and precipitated in ether to yield the desired product as a solid which was then purified by reverse phase chromatography.

High Resolution Mass Spectroscopy

[00219] High-resolution mass spectra were recorded on a Thermo scientific LQT Orbitrap XL under electron spray ionization conditions (ESI) or where indicated under Atmospheric Pressure Ionisation (API) condition.

Circular Dichroism (CD) Spectroscopy

[00220] CD analysis was performed on an Applied Photophysics Chirascan Circular Dichroism spectrometer. Samples were dissolved in D_2O at a maximum of 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

[00221] PMO (91.64 mg, 10 µmol) was dissolved in PBS (5 mL, pH 7.2) and incubated at room temp after the addition of SMCC linker (27.2 mg, 50 µM, 5x excess) dissolved in MeCN/H_2O (1:1, 1 mL). After 30 mins the mixture was desalted using sephadex g25 hydrated in conjugation buffer (PBS 1x, pH 6.8) and was also used as the eluent.

[00222] K1E4-CP8M (17.8 mg, 11.4 µmol) was dissolved in milliQ water (4 mL) and EDTA solution (0.1 mL,100 mM) and mixed with immobilised TCEP (2.5 mL) for 1 h prior. Final concentration of EDTA was 2 mM.

[00223] The freshly desalted SMCC modified PMO had MeCN (8 mL) and EDTA solution (0.1 mL, 100 mM) added before adding peptide the addition of the peptide. The reduced peptide was eluted from the immobilised TCEP into a tube containing the SMCC modified PMO and stirred at RT for 2 hours.

[00224] The solution was loaded on to 3x560 mg HLB columns, and washed with milliQ water to remove any salts, then 10%MeCN in water. When washing with 20% MeCN an amount of PMO was removed from the column. 20% MeCN was sufficient to remove unconjugated PMO from the HLB columns. Columns were washed with 20% MeCN until the eluent ran clear. Finally

PMO conjugate was eluted with 50% MeCN in water. The eluted products then underwent size exclusion chromatography using sephadex superfine g25 hydrated in milliQ water also used as the eluent.

Synthesis of PMO-HP8M and Modification of PMO to PMO-4FB.

[00225] 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. 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 %).

[00226] PMO (30.4 mg, 3 μM) was added to a solution of 4-FB and dissolved in 10 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

[00227] 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.

Cell Culture and Transfection

[00228] HeLa pLuc705 cells were cultured in high glucose DMEM supplemented with 10% foetal calf serum (Sigma, UK) at 37°C under an 8% CO2/92% air atmosphere.

[00229] HeLa pLuc705 cells were setup in 96 well plates with the appropriate dilutions of test compounds either FITC labeled peptides or FITC labeled PMO conjugates diluted into complete culture media (up to 100μM). Cells were then then trypsinised, diluted to $4X10^5$ cells per mL and 100 μL added to each well giving a final volume of 200 μL in each well. Cells were then incubated for either 4 or 24 hours at either 4 or 37 °c.

Flow cytometry

[00230] Uptake of fluorescently-labelled PMO and peptides was determined by flow cytometry using an Accuri C6 flow cytometer. Cells were washed with PBS and glycine buffer then released with trypsin, and kept on ice before analysis in PBS containing 2.5 % FBS. 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, mean fluorescent intensity (MFI) was also calculated. Uptake was determined by gating cells that were able exclude cell-impermanent die (To-pro-3) indicating the ability of cells to retain membrane integrity.

HPLC Analysis

[00231] Samples were run on a Kinetex, 2.6µM particles size, XB-C18 modified with 100 Å pores. Samples were run on a gradient of 0-80% MeCN over 8 mins at a flow rate of 1.5 mL/min at 60°c

Statistical Analysis

[00232] All data are reported as mean values ±SEM or standard deviation as indicated. Statistical differences between treatment groups and control groups were evaluated by SigmaStat (Systat Software, UK) and student's t test was applied or 2 way ANOVA. Significance was accepted for p-values<0.05 using a bonferroni post hoc analysis.

Results

[00233] Solid phase synthesis of stabilised peptides K1E4/5/6-P8M-FITC and the non-cyclised equivalents K1E4/5/6-P8M-NC-FITC, RCM1,5-P8M-FITC and equivalent non cyclised RCM1,5-P8M-FITC-NC, RCM1,5-CP8M and equivalent non cyclised RCM-CP8M-NC and RCM1,5-HP8M was performed with standard Fmoc chemistry on Rink amide resin and yielded the desired product identified by LC-MS in Table 7 below..

[00234] **Table 7**

Sample	Formula	Calculated Molecular mass (Av)	Observed Molecular Mass (Av)
K1E4-P8M-FITC	$C_{89}H_{125}N_{19}O_{21}S$	1829.1510	1829.353
K1E4-FITC-NC	$C_{89}H_{127}N_{19}O_{22}S$	1847.1660	1847.353
K1E5-FITC	$C_{89}H_{125}N_{19}O_{21}S$	1829.1510	1829.353
K1E5-FITC-NC	$C_{89}H_{127}N_{19}O_{22}S$	1847.1660	1847.353
K1E6-FITC	$C_{89}H_{125}N_{19}O_{21}S$	1829.1510	1829.353
K1E6-FITC-NC	$C_{89}H_{127}N_{19}O_{22}S$	1847.1660	1847.353
RCM1,5-P8M-FITC	$C_{92}H_{130}N_{18}O_{20}S$	1840.2180	1840.387
RCM1,5-P8M-NC-FITC	$C_{94}H_{134}N_{18}O_{20}S$	1868.2720	1868.387
RCM1,5-CP8M	$C_{74}H_{124}N_{18}O_{16}S$	1553.9760	1554.150

[00235] HPLC analysis of un-cyclised peptides K1E4/5/6-CP8M-FITC-NC showed elution time of 6.22-6.29 mins using a 0-80% gradient over 8 mins.

[00236] HPLC analysis of cyclised peptides K1E4/5/6-CP8M-FITC showed in increase in elution time over the un-cyclised peptide (Table 8).

[00237] HPLC analysis of RCM1,5-CP8M showed a decrease in retention time of the cyclised product compared to un-cyclised product with a similar retention time to K1E4-CP8M and K1E6-CP8M peptides as illustrated in Table 8 below.

[00238] **Table 8**

Peptide	Not-Cyclised retention time	Cyclised retention time
K1E4	6.295	6.625
K1E5	6.284	6.820
K1E6	6.225	6.571
RCM1,5	6.858	6.723

[00239] Circular dichroism data indicates the solvated structure of K1E4/5/6-CP8M peptides can be influenced by the position of the cross-link (Fig 12).

[00240] K1E4-CP8M exhibits an extended 3_{10}Helix/Poly (pro)II helix with maxima at 219 nm and minima of 196 nm. K1E6-CP8M show similar characteristic maxima and minima (Fig 12).

[00241] K1E5-CP8M displays characteristics of random coil or disordered structure (Fig 12) displayed by the low near 8 elipicity above 210 nm and a minima around 196 nm.

[00242] HeLa pLuc 705 cells incubated in the presence of K1E4/5-CP8M-FITC and K1E4/5/6-CP8M-NC-FITC showed no difference in the uptake of stabilised peptides vs non-stabilised peptides (Fig 13 ai- aii). Surprisingly, K1E6-CP8M did show a significant difference in

uptake compared to the non-stabilised peptide (Fig13 aiii; n = 4, error bars show standard deviation 2 way ANOVA was performed * represents p<0.05, ** represents p<0.001). A 22% increase in FL1+ cells was observed at 11.1 μM when comparing K1E6-CP8M-FITC to the uncyclised control. The difference in FL1+ cells further increased to 43 % difference at 3.7 μM and at the largest difference of 65 % at 1.23 μM and remained constantly higher at all tested concentration. This demonstrates that the position of the cross link that influences cellular uptake is not intuitive between different cyclisation technologies as PCT/GB2016/054028 demonstrates that ring closing metathesis between amino acids a position 1 and 5 within a peptide are efficient at cellular entry.

[00243] HeLa pLuc 705 cells incubated in the presence of K1E4/5-CP8M-FITC and K1E4/5/6-CP8M-NC-FITC demonstrated that a significant increase of over 2 logs in mean fluorescent intensity was only observed for K1E6-CP8M-FITC peptide (compared to it non cyclised control) at concentration greater than 10 μM; Fig 13bi-biii; n = 4, error bars show standard deviation. 2 way ANOVA was performed * represents p<0.05).

[00244] HeLa pLuc 705 cells incubated in the presence of K1E4/5-CP8M-FITC and K1E4/5/6-CP8M-NC-FITC demonstrate no adverse cellular toxicity across all concentration ranges (0.05μM to 100μM; Fig 13ci-iii).

[00245] Hela pLuc 705 cells incubated in the presence of RCM1,5-CP8M-FITC labelled peptide shows a dose dependent uptake of peptide at similar levels to K1E6-CP8M-FITC peptide.

[00246] Hela pLuc 705 cells incubated in the presence of FITC labelled peptides based on charge variants of RCM1,5-CP8M-FITC (Fig14a; *i.e.* RCM1,5-CP8M-FITC 3+, RCM1,5-CP8M-FITC 2+, RCM1,5-CP8M-FITC 1+ and RCM1,5-CP8M-FITC 0+). Fig 14a demonstrated a differential response to peptide dose and cell viability. Data shows that RCM1,5-P8M-FITC-1+ has a significant reduction in FL1+ cells at all concentration below 33 μM compared to other treatments, ranging from 2% difference to 60 % difference of FL1 positive cells. RCM1,5-P8M-FITC-0+ shows in increase in the number of FL1+ cells over a range of concentrations compared to other treatments ranging from a 10% increase to 40 % increase at the largest difference at 0.41 μM (n = 5, error bars show standard deviation. * represents p<0.05, ** represents p<0.001, *a represents an interaction between RCM1,5-P8M-FITC-1+ and all other groups, *b represents an interaction between RCM1,5-P8M-FITC-0+ and all other groups).

[00247] Hela pLuc 705 cells incubated in the presence of FITC labelled peptides based on charge variants of RCM1,5-CP8M-FITC had differential mean fluorescent intensities (Fig 14b). There is no significant difference in the MFI between RCM1,5-P8M-FITC-0+, 1+ and 2+ at a given concentration. However RCM1,5-P8M-FITC-3+ shows an increase in MFI at higher concentrations > 1 μM maximally about 1 log increase (n = 5, error bars show standard deviation. * represents p<0.05, ** represents p<0.001).

[00248] Fig 14c is a comparison of % viable cells judged by gated cells compared to negative controls of HeLa pLuc 705 cells incubated with RCM1,5-P8M-FITC-3+, RCM1,5-P8M-FITC-2+, RCM1,5-P8M-FITC-1+, RCM1,5-P8M-FITC-0+ for 4 hours (n = 3, error bars show standard deviation * represents $p<0.05$, *a represents an interaction between RCM1,5-P8M-FITC-3+ and RCM1,5-P8M-FITC-2+, *b represents an interaction between RCM1,5-P8M-FITC-2+ and RCM1,5-P8M-FITC-0+ and *c represents an interaction between RCM1,5-P8M-FITC-0+ and RCM1,5-P8M-FITC-3+). Data shows that at highest concentration 100 μM a significant loss in the number of viable cells recovered when comparing RCM1,5-CP8M-FITC-3+ to RCM1,5-CP8M-FITC-2+ (32% reduction) and an increase in the number of cells when comparing RCM1,5-CP8M-FITC-2+ to RCM1,5-CP8M-FITC-0+ (20% increase). Similarly at 33 μM RCM1,5-CP8M-FITC-3+ compared to RCM1,5-CP8M-FITC-2+ and RCM1,5-CP8M-FITC-0+ showed a 20 % decrease in the % viable cells. At all other concentration no other significant affects were observed apart from at 0.05 μM. At 0.05 μM RCM1,5-CP8M-FITC-0+ showed an increase of 23 % in viable cells over RCM1,5-CP8M-FITC-3+ and RCM1,5-CP8M-FITC-2+. This has important implications for clinical translation of reduce charge RCM based CPP variants.

[00249] Comparisons of HeLa pLuc 705 cell viability when incubated with either RCM1,5-P8M-FITC-3+ or any of the K1E4/5/6-CP8M-FITC series of peptide demonstrates that at dose ranges 0.05 – 100 μM, that the K1E4/5/6-CP8M-FITC do not have any negative impact on cell viability (Fig 15). Comparing RCM1,5-P8M-FITC to K1E5-P8M-FITC peptides show an increase in viability of 70-99% over the range of concentrations. This has important implications for clinical translation of lactamisation based CPPs.

Conclusion

[00250] From the data generated it can be seen that a CPA stabilized by stapling via alternate cross linking technologies to that the applicant disclosed in PCT/GB2016/054028 are effective at cell entry *in vitro*.

[00251] The importance of position of the crosslink within the peptide sequence has been illustrated as chemistry specific and can greatly influence the solvated conformation of the stabilised peptide and subsequently the cellular uptake as measured by flow cytometry. Thus, surprisingly, it is not intuitive that crosslinks based on different chemical cyclisation technologies, orthogonal to the sequence, generate peptides with either the same conformation or the same cell entry properties. This may be true for other cyclisation chemistries.

[00252] CPAs stabilized by lactamisation cyclisation chemistry stabilize into helical structures and the structures are not α-helical. K1E4-CP8M and K1E6-CP8M exhibits an extended 3_{10}Helix/Poly (pro)II helix structure.

[00253] CPAs stabilized by lactamisation cyclisation chemistry does not cause cellular death *in vitro*. This has important clinical translation implication for DCCPMs based on this technology.

[00254] HPLC analysis of cyclised and non-cyclised K1E4/5/6-CP8M peptides illustrated the similar retention time of non-cyclised peptide 6.22 min (Table 8), however the cyclisation process and resulting stabilised peptides showed an increase in retention times (Table 8). Peptides with inferred conformation of an 3_{10}Helix/Poly (pro)II helix had broadly similar retention times K1E4-CP8M = 6.62 and K1E6-CP8M = 6.57 mins highlighting the potential use of HPLC for identifying changes in conformation.

[00255] Reduced charge variants of CPAs stabilized by a ring closing metathesis cyclisation chemistry are efficient cell entry peptides and have improved toxicological profiles *in vitro*. This has important clinical translation implication for DCCPMs based on this technology.

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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),

    the CPA is a stabilized peptide (CPP) which has a conformation imposed upon it by stapling to form a stapled peptide (StaP) or stitching to form a stitched peptide (StiP), the StiP or StaP comprises a cross link or bridge between at least two amino acids of the peptide and the cross link or bridge provides a cyclisation between the at least two amino acids which are not formed by an olefin metathesis.

2. A DCCPM as claimed in claim 1 wherein cyclisation is achieved by one or more of:
    i. condensation of an aldehyde or ketone with a hydrazine or protected hydrazine;
    ii. a thiol-ene Michael addition;
    iii. a di-sulfide formation;
    iv. a Huisgen 1, 3 di-polar cycloaddition;
    v. a reaction between an amine and carboxylic acid;
    vi. a singlet or triplet based carbine reaction; or
    vii. a Suzuki or Sonogashira coupling.

3. A DCCPM as claimed in claim 2 wherein in iv) a triazole is formed between an azide or electron deficient nitrile containing amino acid and a propygyl containing amino acid.

4. A DCCPM as claimed in claim 3 wherein the azide is azidolysine.

5. A DCCPM as claimed in claim 3 wherein the propygyl containing amino acid is lysine, glutamic acid or aspartic acid.

6. A DCCPM as claimed in claim 2 wherein in v) a lactam is formed between a free amine containing amino acid and a carboxylic acid containing amino acid.

7. A DCCPM as claimed in claim 6 wherein the lactam is formed by cross linking a lysine and glutamic or aspartic acid.

8. A DCCPM as in any of the preceding claims wherein each crosslink has a nominal sequential length of from 2-20.atoms.

9. A DCCPM as claimed in any of the preceding claims wherein the stabilized peptide comprises at least one alpha helix, extended 3_{10}-helix or poly (Pro) II helix.

10. A DCCPM as claimed in any of claims 1 to 8 wherein the stabilized peptide comprises at least one beta sheet or hairpin or turn.

11. A DCCPM as claimed in claim 9 or 10 wherein the stabilized peptide comprises at least one alpha helix, extended 3_{10}-helix or poly (Pro) II helix and one beta sheet, turn or hairpin.

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

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

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

15. A DCCPM as claimed in claim 13 or 14 wherein the ON is a peptide nucleic acid (PNA) or a phosphorodiamidate morpholino oligonucleotide (PMO) or variants thereof.

16. A DCCPM as claimed in any of claims 12 to 15 wherein the ON is an anti-sense oligonucleotide (AO).

17. A DCCPM as claimed in claim 16 wherein the ON is an anti-sense oligonucleotide comprising a chemistry selected from the compounds of Table 4, including ON that have hybrid chemistries.

18. A DCCPM as claimed in claim 12 wherein the BAC is a phosphorodiamidate morpholino oligonucleotide (PMO).

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

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

21. A DCCPM as claimed in any of the preceding claims wherein the BFL comprises a chemistry selected from the chemistries of Table 6.

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

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

24. A DCCPM as claimed in claim 21 wherein the BFL is HNA and has been incorporated into the terminal end of the CPP.

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

26. A DCCPM as claimed in claim 23 wherein the SMCC is PEGylated.

27. A DCCPM as claimed in claim 24 of 25 wherein the HNA is PEGylated

28. A DCCPM as claimed in claim 21 wherein the CPA is linked to a first end of the BFL covalently.

29. A DCCPM as claimed in claim 21 wherein the BAC is linked to a second end of the BFL covalently.

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

31. 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), the StiP or StaP comprises a cross link or bridge between at least two amino acids of the peptide and the cross link or bridge provides a cyclisation between the at least two amino acids which are not formed by an olefin metathesis, 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.

32. A DCCPM as claimed in any of claims 1-30 for use in the treatment of a disease requiring alteration of the expression of an endogenous or exogenous gene.

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

34. A DCCPM as claimed in claim 32 for use in the treatment of Duchenne's muscular dystrophy.

35. A DCCPM as claimed in claim 34 wherein the DCCPM comprises an AO targeting the dystrophin gene.

36. 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) and the StiP or StaP comprises a cross link or bridge between at least two amino acids of the peptide and the cross link or bridge provides a cyclisation between the at least two amino acids which are not formed by an olefin metathesis.

37. 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 the StiP or StaP comprises a cross link or bridge between at least two amino acids of the peptide and the cross link or bridge provides a cyclisation between the at least two amino acids which are not formed by an olefin metathesis and administering it to a subject.

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

39. 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 1-30 or 32-35.

40. A composition comprising a DCCPM as claimed in any of claims 1-30 or 32-35 and one or more pharmaceutically acceptable excipients.

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

Intellectual Property Office

Application No:	GB1710322.7	**Examiner:**	Dr Bill Thomson
Claims searched:	1-41	**Date of search:**	23 March 2018

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X,Y	X: 1, 2, 8, 12-23 & 30-41 at least Y:1-23 & 30-41 at least	WO 2016/187425 A1 (SAREPTA THERAPEUTICS) - See whole document, in particular page 2, line 2 - page 5, line 8; page 60, line 3-page 68, line 11, Examples 3-5 and Figures 3B-3D
Y	1-23 & 30-41 at least	"Stitched alpha-helical peptides via bis ring closing metathesis", G.J. Hilinski et al, J. Am. Chem. Soc., (2014), 136, 12314-12322 See whole document, in particular the abstract, page 12314, Column 2, lines 2-5 and Table 1
Y	1-23 & 30-41 at least	WO 2017/011820 A2 (PROTAGONIST THERAPEUTICS INC.) - See whole document, in particular paragraphs 31, 358, 546, 613, 614 and Figure 10

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
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Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC^{X} :

Worldwide search of patent documents classified in the following areas of the IPC

A61K

The following online and other databases have been used in the preparation of this search report

BIOSIS, CAS-ONLINE, MEDLINE, PATENT FULLTEXT & WPI

International Classification:

Subclass	Subgroup	Valid From
A61K	0047/64	01/01/2017
A61P	0021/00	01/01/2006
C07K	0001/107	01/01/2006