Peptide-Oligonucleotide Hybrids in Antisense Therapy

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Abstract: Antisense technology provides outstanding promise for treatment of human disease, having broad applicability and high specificity. Although advances have been made in antisense oligonucleotide chemistry, leading to increased plasma and cellular stability, and decreased toxicity, considerable potential remains for the enhancement of oligonucleotide uptake for targeted delivery of oligonucleotides. One promising avenue for achieving this is *via* linkage of antisense oligonucleotides to peptide carriers. This review looks at the current status of developments in this area.

ANTISENSE THERAPY

Antisense therapy holds much promise for the treatment of various disorders. With the approval several years ago of "Vitravene" for the treatment of cytomegalovirus-induced retinitis and the increasing list of compounds in phase II and III clinical trials (see [1]), the potential clinical applications for antisense therapy appear endless. Antisense oligonucleotides bind with Watson-Crick hydrogen bonding to a target mRNA within hybridisation-accessible sites and can thus down-regulate the expression of disease-causing proteins by inhibiting gene expression at the level of mRNA. In theory, antisense therapy allows for the rational design of highly sequence-specific nucleic acid drugs that can target and even destroy a given mRNA. However, one of the major obstacles for antisense therapy is efficient delivery to, and uptake into, target cells.

Over the years, the chemical backbone of antisense oligonucleotides has been modified to confer or enhance nuclease resistance so that the oligonucleotide will remain intact longer and thus reach the intracellular target to produce an antisense effect. Changing backbone chemistry can also change the mechanism of antisense effect from RNase H-dependent (phosphodiester, phosphorothioate) to RNase H-independent (morpholino and 2'-*O*-methyl), whereas "gapmers" provide hybrids between different chemistries to combine functionalities from both (eg. RNase H activation with nuclease resistance). An alternative to the traditional phosphodiesters and phosphorothioates or the newer morpholino and 2'-*O*-methyl backbones is peptide nucleic acids (PNA). PNAs are synthetic DNA analogues in which the sugar phosphate backbone is replaced with a 2-aminoethyl-glycine linkage giving rise to a flexible, uncharged backbone; while this chemistry confers enhanced stability and resistance to nucleases and proteases, delivery still poses a major problem due to poor membrane permeability (for reviews see [2, 3]).

Nuclease resistance does not confer efficient delivery to the target site. For antisense therapy to be effective, the antisense oligonucleotide must distribute to the target organ and be taken up by target cells. Following intravenous administration, however, the greatest accumulation of oligonucleotides is found in the liver and kidney for mice [4, 5] and rats [6]. Modified oligonucleotides, 2'-O-(2-methoxyethyl) (2-O-MOE), also accumulate in liver and kidney, although the improved stability may slow clearance of the oligonucleotide from target tissue/cells [7]. The brain, however, appears to be the greatest barrier for antisense penetration. While low level entry of phosphorothioate antisense oligonucleotides into the brain of mice has been reported [5], uptake is poor in comparison to other tissues. High accumulation in clearance tissues such as liver is only advantageous if this is the target organ (for example [8]), however in the many instances where the liver is not the organ of interest or the oligonucleotide cannot be directly administered to the target organ either by inhalation or intravitreal administration, mechanisms for delivery enhancement or targeted delivery have potential to significantly improve the utility of antisense-based agents. One mechanism to enhance delivery (both into cells and into the nucleus) or achieve targeted delivery is by conjugation of the nucleic acid to an appropriate peptide.

CHEMISTRY OF PEPTIDE–OLIGONUCLEOTIDE CONJUGATES

Considerable work has been directed towards the solution of technical difficulties underlying the conjugation of peptides to nucleic acids, encompassing 5' versus 3' coupling of oligonucleotide to either the N- or C-terminus of peptides. Strategies for generation of such differing variants using assorted linkages are outlined below. However, while study of the technical aspects of generation of peptide-oligonucleotide hybrids is well advanced, detailed exploration of sequence and structural requirements for efficient cell penetration and compartmentalisation of hybrids and subsequent correlation with biological activity remains to be addressed. The following section reviews approaches for generation of peptide-oligonucleotide hybrids.

There are a number of methods for the preparation of peptide-oligonucleotide conjugates: post-synthetic conjugation (or post-assembly conjugation, fragment coupling strategy), total stepwise synthesis (or on-line solid-phase synthesis), native ligation and template-directed ligation. Advantages and disadvantages are associated with each method.

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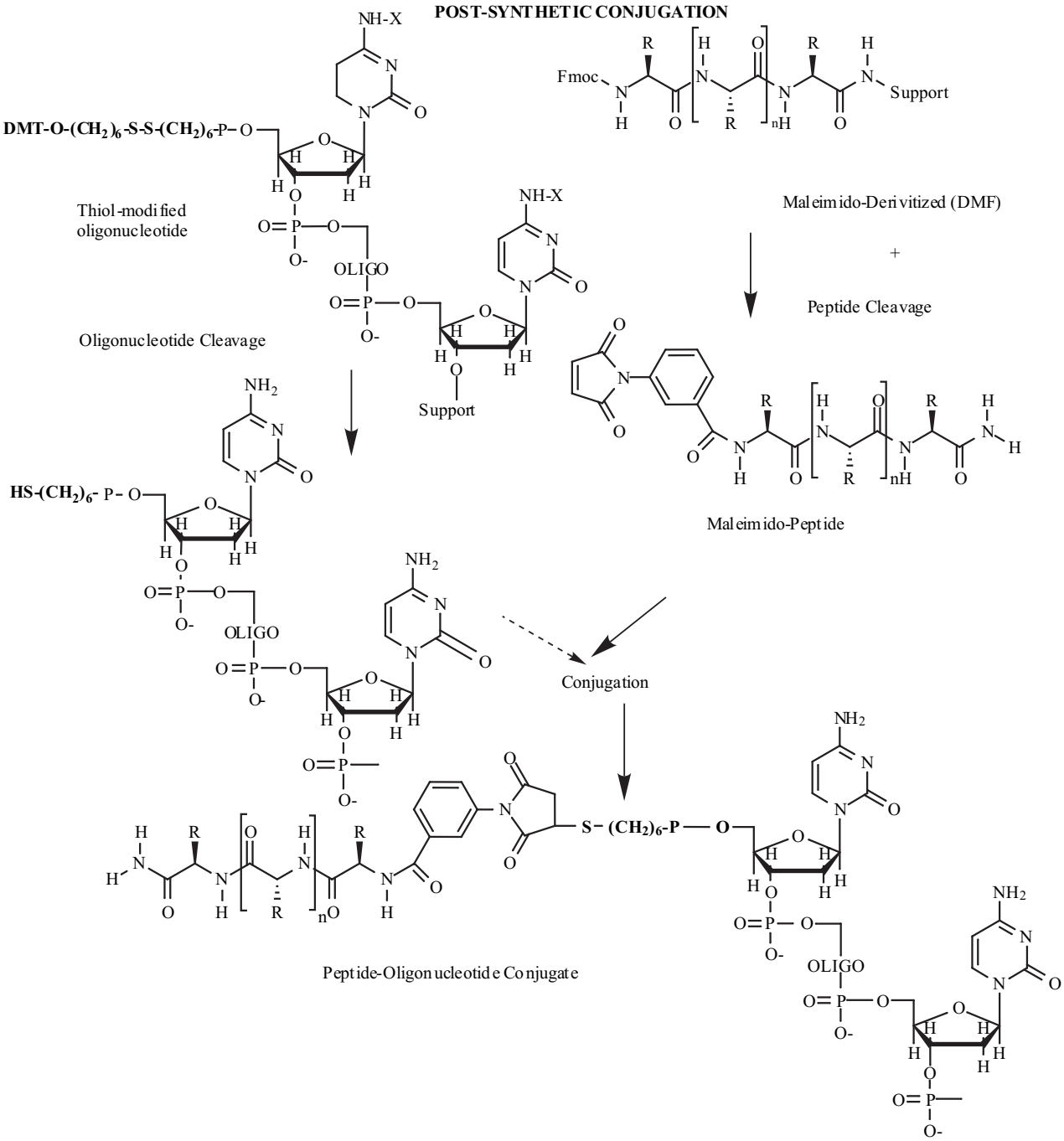

Fig. (1). Schematic of post-synthetic conjugation. Peptide is modified with a maleimido group and cleaved from supporting resin. Oligonucleotide is modified with a 5' thiol group and cleaved from support. Conjugation between maleimido and thiol groups occurs post synthesis to form the oligonucleotide-peptide conjugate. Modified from de la Torre (1999) [10].

(i). Post-Synthetic Conjugation

In this strategy, oligonucleotide and peptide are prepared separately on their own supports, with each designed to carry reactive functionalities such as thiols on the oligonucleotides and maleimido groups on the peptides, to allow later conjugation [9, 10]. The specific functional group (primary amine or thiol) to conjugate to the peptide is generated when the oligonucleotide is cleaved from the support. Generally, the peptide remains N_{α}Fmoc protected until removal from its support. The conjugate is generated from the reaction of the free thiol of the oligonucleotide with the activated maleimido of the peptide, (Fig. **1**).

Thiols afford specific and rapid conjugation and react with a wide variety of substrates [11]. In addition, conjugation to maleimido peptides gives rise to better results as there are fewer side products [12]. Thiols have been incorporated into the 5' end of the oligonucleotide and conjugated to a N-terminal maleimido peptide [9, 10, 12, 13], to a C-terminal free sulfhydryl peptide [14], to a N-terminal N_{α} bromoacetyl-derivatised peptide [15] or to a protein bearing pyridyl dithiohexanoyl groups *via* a disulfide

bond [16]. The latter conjugation produced six antisense molecules per protein. Conjugation *via* the 3' end of the oligonucleotide is also possible, although more difficult; 3' thiols have been conjugated to a maleimido peptide [11]. An advantage to the latter conjugation is that ligation of a peptide to the 3' terminus of the oligonucleotide will also protect it from 3' exonucleases [11].

An alternative to incorporating a thiol is the functionalization of the 5' end of the oligonucleotide with a primary amino group for conjugation to Fmoc-peptides [17]. One example of this conjugation procedure was reaction of a 5'aminohexyl-modified oligonucleotide with thiol-containing nuclear localisation sequences (NLS). Attachment of NLS peptides had no adverse effect on hybridisation of the oligonucleotide to DNA, but did lead to loss of antisense activity. However, in this case, the loss of antisense activity was dependent on the peptide sequence used, with random peptide-DNA hybrids maintaining efficacy [18]. It was proposed that the NLS peptides may have been unable to release from a nuclear transport receptor or delivered the oligonucleotides to a region where they were unable to interact with mRNA. Other work with conjugates of a NLS to oligonucleotides, revealed a significant decrease in hybridisation of the hybrid to RNA, and was suggested to be related to ability of the hybrid to invade secondary structure [10]. However, given that antisense activity could be recovered in hybrids with random peptides [18], it is probable that other factors, such as the highly basic nature of the NLS, also contributed to loss of antisense efficacy.

Functionalization of oligonucleotides can also be achieved *via* incorporation of aminooxy groups thereby avoiding some of the potential problems of thiol linkages. 2'-O-methyl ribonucleotides have been functionalized in this manner using 4-(2-aminooxyethoxy)-2-(ethylureido) quinoline (AOQ) and 4-ethoxy-2-(ethylureido)quinoline (EOQ) to generate oligonucleotides with high reactivity towards ketones and aldehyde groups [19]. Formation of the AOQ-oligomer resulted in increased target hybridization efficiency compared to oligonucleotide alone. This was proposed to be a result of the quinoline ring of the AOQ (or EOQ) group having the capacity to stack on the last base pair formed between the oligomer and target, thus increasing stability of the duplex [19]. The AOQ/EOQ-modified oligonucleotide can be conjugated to a ketone group introduced into the peptide *via* bromoacetone treatment forming a highly stable oxime linkage. Conjugation of aminooxy oligomers with leupeptin or Human immunodeficiency virus (HIV) trans-acting transcriptional activator (Tat)-derived keto-peptides was successfully achieved using this method but no functional studies were performed to assess the cellular delivery of the oligonucleotide [19].

Post-synthetic conjugation generally requires a molar excess of peptide to oligonucleotide. Optimal conjugations, have been reported using a 2-fold excess [15], a 3-fold excess [14], a 5-fold excess [17], a 10-fold excess [9, 12, 17] and a 10-15-fold excess [11], suggesting that optimal conditions vary according to peptide sequence and chemistry and needs to be determined empirically. Peptides conjugated to oligonucleotides using this strategy are included in Table **1**. In contrast, Zatsepsin *et al.* [20] has described linking peptides containing cysteine, aminooxy or hydrazide groups to aldehyde-containing oligonucleotides to form thiazoline oxime-only hydrazone linkages [20]. The advantages of this method were described as three-fold; the reaction did not require a large excess of peptide (1.2-1.8 equivalents); there is an ability to attach more than one peptide to oligonucleotides at defined sites and the freedom to attach additional moieties i.e., fluorescent groups at both the 3' and 5' ends of the oligonucleotide. In addition, linkage by this process did not interfere with oligonucleotide-to-target hybridization.

A major advantage to the post-synthetic approach is that peptides do not need to be stable under conditions of oligonucleotide assembly or vice versa thus, unlike the total stepwise approach (see below), there is no issue of incompatibility of deprotection and assembly chemistries; all peptide side chains and nucleobases are deprotected before conjugation. There are, however, several potential disadvantages to this method. For example, highly basic peptides form non-specific interactions with oligonucleotides, which may lead to poor coupling efficiency and low yield. Where a disulfide linkage is utilised, this may be unstable to reducing agents present in the assay or cell environment (although in some instances this may be a preferred option). For maleimido-thiol linkage the conjugation reaction can be inefficient and presence of a terminal peptide cysteine residue is required along with an additional functionalization step on the oligonucleotide (see [21]).

(ii). Total Stepwise Solid-Phase Approach

In the total stepwise method, peptide-oligonucleotide conjugates are prepared by the stepwise addition of amino acids and nucleobases in solid phase on a single solid support, (Fig. **2**) [22, 23]. With this strategy, however, the choice of protecting group or the right combination of protecting groups on nucleosides and amino acids is a critical factor. Difficulties also arise from incompatible chemistries for deprotection and present another barrier to obtaining a substantial yield. The chemistries used need to be mild enough to support the consecutive synthesis of oligonucleotide and peptide and the deprotection conditions must not affect the oligonucleotide. Thus, the preparation of oligonucleotide-peptide conjugates in sufficient amounts for *in vitro* analysis is limited to the synthesis of peptides containing residues without acidolytic deprotection; deprotection of a peptide by acidolysis may induce depurination of the oligonucleotide [11]. Amino acid protecting groups need to be chosen to allow efficient elongation of the growing chain and easy removal of the protecting groups under mild basic or acidic conditions in order to safely deprotect the oligonucleotide [24]. Thus there is a limit to the number of conjugates that can be prepared due to the incompatibility of the peptide and oligonucleotide protecting groups. Although this can be overcome by synthesising the peptide and oligonucleotide separately in solid phase and derivatising with functionalities that are mutually reactive (see post-synthetic strategy, above), it is an attractive ideal to synthesise peptide and oligonucleotide as one with the ultimate aim of fully automating the synthesis of peptide-oligonucleotide conjugates.

Table 1. Peptides Used in Various Chemical Approaches to Peptide-Oligonucleotide Conjugation

Peptide	Conjugation chemistry	Reactive groups/conjugation	Reference
(i) PTSQSRGDPTGPKE (ii) DRVIEVVQGAYRAIR-NIPRRIRQG	Post-synthetic approach	Maleimido-derivatised peptide to a 3' cysteine derivatised ON.	[11]
(i) LARLLARLLARL (ii) TQPREEQYNSTFRV	Post-synthetic approach	N-terminal maleimido to a 5'thiol ON.	[9]
AAPKKKRKV	Post-synthetic conjugation	N terminal cysteine or maleimido-derivatised peptide to a 5' thiol derivatised ON.	[10, 12]
CTPPKKKRKV	Post-synthetic approach	Thiol-containing peptide to a 5'aminohexyl ON	[18]
QAKKKKLDK	Total stepwise conjugation	3' ON to a C-terminal peptide.	[10]
(i) CNSAAFEDLRVLS (ii) MNKIPIKDLLNPQFC	Post-synthetic approach	Thiol-containing peptide to a 5'aminohexyl ON.	[18]
GRKKRRQRRRPPQC	Post-synthetic approach	C terminal free sulfhydryl group to a 5' thiol	[14]
(i) LGIG (ii) QRRRPPQG	Post-synthetic approach	C terminal peptide to a 5' amino ON; increasing linker length improves yield.	[17]
KDEL	Post-synthetic approach	N-terminal bromoacetyl derivative of peptide to 5'thiol; 3'amino used for additional conjugation of fluorescein	[15]
Tyr ^{3}-octreotate	Post-synthetic approach	N terminal maleimido to 5' thiol	[13]
Asialoglycoprotein (ASGP)	Post-synthetic approach	Protein covalently linked to 5' thiol ON (6 ONs per ASGP)	[16]
(i) AVGAIGALFLGFLGA-AG (ii) ALFLGFLGAAG	Total stepwise approach	3' end of the ON to N-terminus peptide	[22]
D-GCSKAPKLPAALC	Total stepwise approach	3' conjugation; branched linker for extension of ON from hydroxyl arm and peptide from amino arm.	[30]
D-CSKC	Total stepwise approach	PNA-peptide conjugate with $(Gly)_4$ spacer	[39]
GGH	Total stepwise approach	3'conjugate with aminohexanol linker	[36]
Tetrapeptide of all but two naturally occurring amino acids (Arg, His).	Total stepwise approach	5' conjugate; linker free conjugate	[29]
KKK	Total stepwise approach	3' end of the ON to N-terminus peptide	[27]
AAVALLPAVLLALLAP (*Kaposi* fibroblast growth factor)	Total stepwise approach; assessed 3 different solid supports.	3' end of ON linked to C terminus of peptide *via* PO or PS bond	[28]
(i) MYIEALDKYAC (ii) MHIESLDSYTC	Total stepwise approach	3' conjugate; linker between ON and peptide	[35]
(i) W-ON-AFG (ii) T-ON-AFG (iii) S-ON-AFG (iv) homoserine-ON-AFG	Total stepwise approach	Single amino acid at the 3' end of ON to confer exonuclease protection; stability to 3' exonucleases Thr>Ser> homoserine>Tyr	[23]
$(Ala)_6$	Total stepwise approach; further elongation to form DNA-peptide-DNA hybrid	5' conjugate; no linker required.	[34]
(i) KGH (ii) HGH	On-line synthesis	3' end of the ON to N-terminus peptide	[24]
$(LKLK)_3$	On-line synthesis	3' end of the ON to N-terminus peptide	[26]
AAVALLPAVLLALLAPC	Stepwise solid-phase synthesis	3' end of the ON linked to the C-terminus of a peptide by PS or PO linkage	[41]
(i) PTSQSRGDPTGPKE (ii) Sar-Leu-Gly-Ile-Gly (iii) ALPPLERLTL (iv) GALFLGFLGAAGST-MGAWSQPKSKRKV	Native ligation	N-terminal thioester peptide was conjugated to a 5'-cysteinyl oligonucleotide.	[21]
(i) AAKRVKLG (ii) WGGFLRRG (dynorphin)	Template-directed ligation	Joined by stable amide bond; 3' conjugate to C terminal peptide	[40]

ON: oligonucleotide; PO: phosphodiester; PS: phosphorothioate

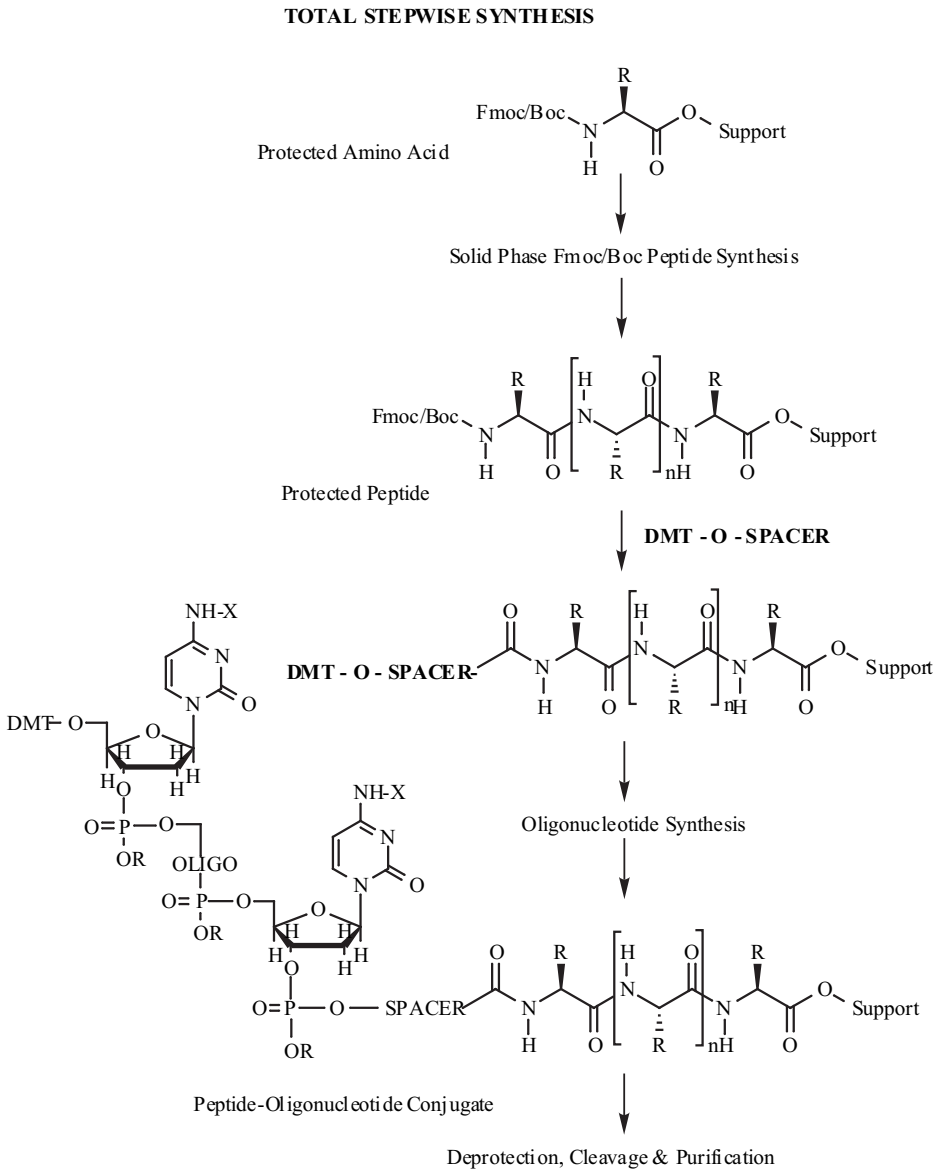

Fig. (2). Schematic of total stepwise synthesis. Peptide is synthesized on support using standard Boc or Fmoc chemistry and modified with DMT. Oligonucleotide is subsequently synthesized onto peptide and complete oligonucleotide-peptide conjugate is cleaved from the support. Modified from de la Torre (1999) [10].

Conjugations using this method primarily link the 3' end of an oligonucleotide to the N-terminus of the peptide [22, 24-27]. However, the 3' terminus of an oligonucleotide has been conjugated to the C-terminus of a peptide [28], and 5' peptide-oligonucleotide hybrids have also been prepared [29]. While conjugation to the 3' end of the oligonucleotide provides nuclease resistance, additional protection from degradation can be conferred through linkage to a D-peptide, which engenders protection against the action of cellular proteases [30]. The various peptides that have been used to conjugate to antisense oligonucleotides with this strategy are included in Table **1**.

Although in most instances biological assessment of antisense activity has not been performed, thermal denaturation studies have demonstrated that the 3' linked peptide moiety does not interfere with the DNA hybridisation efficiency of the oligonucleotide [22]. In addition, conjugation of the NLS derived from nucleoplasmine, QAKKKKLDK, to an oligonucleotide can enhance the affinity of the oligonucleotide to complementary DNA, but lower the affinity (approximately 10-fold) for the target RNA [10]. Enhancement of DNA-binding in this case is thought to be related to the highly basic nature of the peptides [10, 31-33]. Nonetheless, the discrepancy between DNA melting temperature and RNA-hybridisation indicates potential limitations in using DNA-binding as a marker of RNA-hybridisation potential.

With total stepwise solid phase synthesis, it is necessary to prepare a suitable solid support for conjugate synthesis and avoid unwanted side reactions. Generally this method requires the preparation of ad hoc-derivatised supports that link a suitable spacer, which can be selectively cleaved at the end of conjugate synthesis. Many of these methods start with the synthesis of the oligonucleotide. The support used

for this method varies between groups. Silica supports of controlled pore glass (CPG) or Fractosil derivatised with 1-9-hydroxydecanoic derivative to introduce a spacer arm with a terminal hydroxy group have been used successfully [26]. However, others have found that CPG is not efficient for peptide synthesis and have used instead *O*-nitrophenyl polyethylene glycol polystyrene supports [25]. Other supports include sarcosine-modified CPG support with an allyl phosphate as the protecting group on the last coupling (for compatibility with Fmoc chemistry) [34], a base-labile bridge to a solid matrix-long chain alkylamino CPG [35], polymeric supports [36], or polyethyleneglycol-polystyrene [10, 30].

As alluded to above, difficulties in stepwise synthesis can arise from incompatible chemistries for peptide and oligonucleotide protection and assembly. Consequently, this approach has been limited to incorporation of peptides with amino acids with side chains compatible with base-labile protecting groups or those amino acids that do not require protection. Base-labile protecting groups are available for the side chains of lysine, aspartic acid and glutamic acid, however a suitably protected arginine derivative is neither available nor easy to prepare. However arginines are prevalent in peptide sequences designed to enhance plasma membrane penetration and/or nuclear localisation, leading to restrictions in the current utility of this approach. To overcome lack of a suitable protecting group Antopolsky and colleagues proposed a process using Fmoc-ornithine-methyltrityl (Mtt) as a precursor for arginine. Conversion of ornithine to arginine required additional steps which involved treatment of the peptide containing Fmoc-Orn(Mtt) with TFA/dichloromethane, triethylamine/dichloromethane and finally incubation in THF/triethylamine [37]. The base-labile protecting groups Fmoc and Fm have been used for lysine and aspartic acid, respectively which are removed using concentrated ammonia after detritylation [10, 38]. This method avoids the use of strong acids (e.g., TFA) for removal of tBu groups that will cause depurination of DNA after conjugation of peptide with oligonucleotide.

Oligonucleotide backbone chemistry can also be exploited to aid the preparation of conjugates. Where a PNA antisense molecule is ligated, synthesis of the hybrid is easier as the PNA can be directly extended from the peptide sequence. For example, Basu *et al.* [39] assembled a peptide-PNA hybrid as a continuation of an Fmoc-protected peptide with manual coupling of the Boc–protected PNA monomers. In this case a Gly-Gly-Gly-Gly[1] spacer was incorporated between the principle peptide sequence and the PNA to minimise mutual interference.

The primary advantages of the total stepwise method over the post-synthetic approach is that total stepwise solid-phase procedures require only one purification step after final deprotection and are compatible with automation. While not well studied, one comparison of post-synthetic and total stepwise approaches for yield and purity found that both strategies produced the desired peptide-oligonucleotide conjugate in similar yields and purity [10], although the comparison in this instance was not ideal with a 5' thiol oligonucleotide being linked to a N-terminal maleimido peptide using the post-synthetic approach whereas the conjugation using the total synthesis method was to the 3' end of the oligonucleotide.

(iii). Native Ligation

While both the post-assembly and total stepwise methods of conjugation have their advantages, they are not without their limitations (see [21]). The method of native ligation arose out of a search for an alternative method for conjugate formation [21]. In this procedure peptide and oligonucleotide are first synthesised separately on solid supports. The unpurified, deprotected, functionalised peptide and oligonucleotide can then be used in a native ligation conjugation in aqueous solution, (Fig. **3**). Native ligation has the advantage that efficient coupling is achieved in the presence of denaturing agents and organic solvents. Thus in principle, this method is suitable for both hydrophobic and hydrophilic peptides (see [21]). In the method described by Stetsenko and Gait [21], an N-terminal thioester peptide was conjugated to a 5'-cysteinyl oligonucleotide. The peptides used to conjugate to oligonucleotides are included in Table **1**. Successful peptide–oligonucleotide conjugation occurred when amino acid cyclization side reactions could not take place, as was the case for peptides with an N-terminal proline, sarcosine or alanine. In contrast, a peptide with an N-terminal glycine was prone to cyclization and thus no conjugation product was formed [21].

(iv). Template-Directed Ligation

An oligonucleotide template can be used to direct the ligation of peptides to oligonucleotides *via* a stable amide link allowing ligation of unprotected peptides to oligonucleotides in aqueous solution. An example of this method is illustrated by Bruick *et al.* [40] who converted a C-terminal thioester peptide to a thioester-linked oligonucleotide-peptide intermediate; the oligonucleotide portion bound to a complementary oligonucleotide template, placing the peptide in close proximity to an adjacent template-bound oligonucleotide that terminated in a 3' amine. The ensuing reaction results in an amide-linked oligonucleotide-peptide conjugate. As with the native ligation procedure, amino acid cyclization can be a problem, thus certain small peptides may adopt a cyclic conformation where the amine terminus of the peptide competes with the amine-terminated oligonucleotide resulting in cyclization of the peptide rather than ligation of the peptide to the oligonucleotide. Peptides that have been used in template-directed ligation are included in Table **1**.

GENERAL CONSIDERATIONS FOR CONJUGATION CHEMISTRY

(i). Where to Conjugate?

Peptides and oligonucleotides can be linked *via* either the N- or C-terminus or the 3' or 5' end, respectively. While it has been suggested that conjugation *via* the 3' end of the oligonucleotide affords exonuclease resistance to the oligonucleotide, there are currently almost no studies that directly examine the relative advantages or disadvantages of

[1]Three letter code used for up to 6 amino acids. Larger sequences are listed using single letter coding.

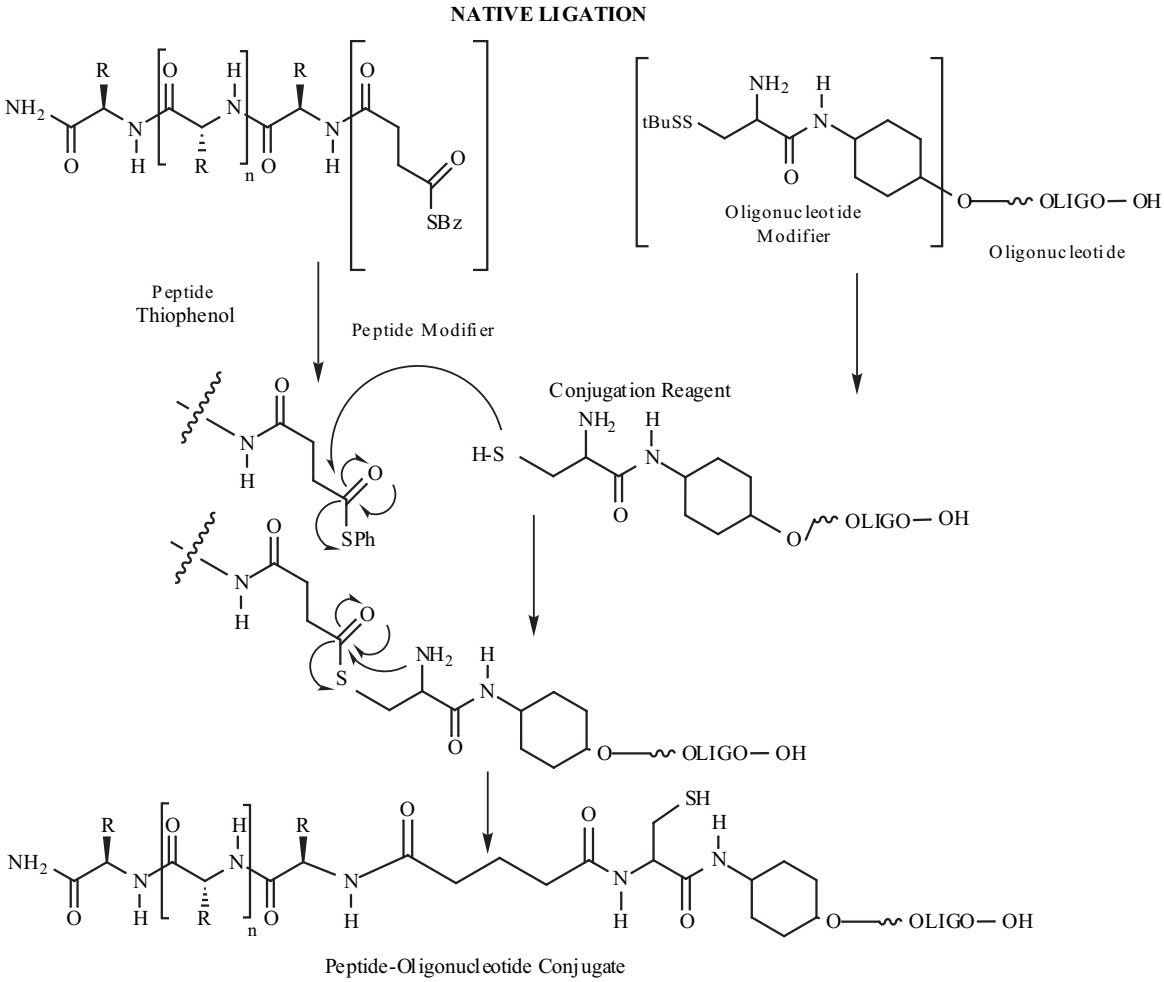

Fig. (3). Schematic of native ligation based on OPeC^{TM} peptide-oligonucleotide conjugation reaction. Peptide is synthesized using routine chemistry, functionalized with peptide modifier and purified from support. Oligonucleotide is synthesized separately, modified without purification and conjugated with modified peptide in a separate reaction. Reproduced with permission from Link Technologies. OPeC^{TM} reagents for research use are exclusively licensed to Link Technologies Ltd (**www.linktech.co.uk**) under Patent No. PCT/GB00/03306 from the Medical Research Council, London, UK.

coupling *via* the N- or C-terminus of the peptide on hybrid molecule activity. In one study, where the 3' terminus of a phosphorothioate oligonucleotide was disulfide coupled to either the C- or N-termini of a membrane permeable peptide, little difference in luciferase knockdown activity between the two conjugates was observed [41]. It is likely that the choice of peptide linkage site will depend on the required functionality imparted by the peptide and that in most cases the effect of conjugation on this activity will have to be determined empirically.

(ii). Linkage Chemistry

As oligonucleotides are synthesised 3' to 5', it has been necessary to develop appropriate linkages between the solid support and the 3' end of the first nucleotide. Linear 3' linkers are useful for post-assembly conjugations, where the oligonucleotide is cleaved from the support. In contrast, branched linkers provide greater synthetic flexibility. Branched linkers allow the incorporation of the conjugation molecule before oligonucleotide assembly, or the oligonucleotide can be assembled first and post-assembly conjugation can be carried out after release into solution, or the functionality can be used as an attachment point for solid-phase conjugation (see [27] and [35]). With a branched modifier linked to the support, the two arms, carrying two different groups, allow flexibility to extend the DNA sequence from the hydroxyl arm and the peptide sequence from the amino arm [30].

To improve efficiency of conjugation and/or maintain biological efficacy of the hybrid the incorporation of a spacer between oligonucleotide and peptide may also be necessary. This may serve a number of functions. For highly basic peptides, a rigid spacer may improve both the yield and efficacy of products; providing distance between the bulk of the peptide and the oligonucleotide and thus preventing non-specific binding of the peptide and oligonucleotide during conjugation while decreasing the capacity for the conjugated peptide to fold back on the oligonucleotide and inhibit its antisense action. The optimal length of an alkane spacer has been investigated in at least one study where a 6 carbon chain provided improved yields for conjugates containing the Leu-Gly-Ile-Gly or HIV Tat peptides [17]. While others have used a 9-carbon spacer arm between a tripeptide and an antisense oligonucleotide [24], six-carbon spacers currently appear to be the preferred option, albeit without a lot of empirical investigation into the optimal spacer length.

Spacers may, alternatively, be required to allow conformational flexibility of the resulting hybrid molecule. In the conjugation of the protein, asialoglycoprotein to an antisense oligonucleotide, the bifunctional cross linker (succinimidyl 6-[3'-(2-pyridylthio)propionamido] hexanoate) provided a spacer arm to allow the conjugate sufficient conformational flexibility for the terminal galactose residues to bind to the asialoglycoprotein receptor [16]. This consideration is likely to be relevant to other peptides that target high affinity binding sites as their mechanism of action.

Consideration of the use of "spacers" also extends to peptide design and may be particularly important where incorporation of dual functionality through combination of peptide motifs is desired. An example of this arises from work with hybrids based on peptides integrating a signal sequence (to enhance membrane permeability) and a NLS (to target the oligonucleotide to its primary site of action). In this situation a spacer sequence (Trp-Ser-Gln-Pro) was required to allow efficient targeting of the peptide to the nucleus by the NLS sequence [42], indicating that conformational flexibility between the two motifs was required for full functionality.

The choice of peptide used in generation of the hybrid molecule will also influence efficiency of conjugation and subsequent yields. Peptides such as Tat(43-60) and Antennapedia (Ant)(43-58) are highly basic and while functionality associated with these sequences (i.e. cell penetration and nuclear localisation) may be highly desirable, they may also form strong ionic interactions with negatively charged DNA. This presents problems for the generation of hybrids with DNA including precipitation of complexes and low yields. Nonetheless, a number of groups have described modifications to standard reaction protocols to address these problems including alterations to salt and acetonitrile concentrations and report reasonable yields [14]. While not the subject of this review, other groups have utilised the non-specific DNA binding properties of basic peptides to produce DNA "condensing" peptides that have been used as an alternate approach to enhancing oligonucleotide uptake [43].

(iii). Covalent Versus Metabolically Labile Attachment

Due to the simplicity of its chemistry, peptide-oligonucleotide hybrids are commonly linked *via* a disulfide bond. These linkages may be broken under reducing conditions that can occur in the intracellular environment. While, this may lead to reduced stability of hybrid molecules [21], there may also be advantages to incorporation of a metabolically labile linkage. Where peptides improve cellular uptake but do not specifically enhance nuclear localisation of hybrids, release of oligonucleotide in a "pro-drug" strategy could lead to improved efficacy. Such rationalisation was utilised in hybrid design by Vivès and Lebleu [14] with the highly basic peptide derived from HIV Tat and Rajur *et al.* [16] with asialoglycoprotein. Nonetheless, direct comparison of covalent versus disulfide linkage has not been performed, and indeed the degree to which cleavage occurs is not well studied. *In vitro* analysis of one series of peptide-oligonucleotide hybrids suggested very little cleavage of hybrids occurred within CV1-P fibroblast cells over a 4h incubation period [41], although this may be related to the endosomal "trapping" of hybrids observed in the study. For covalent linkage, the choice of chemistry may also influence the efficacy of the hybrid. This is exemplified in a study by Eriksonn and colleagues who demonstrated 10-fold lower efficacy with maleimide versus ethylene-glycol linked peptide-PNAs [44], and may be related to the degree of conformational flexibility provided by the different linkers.

BIOLOGICAL ACTIVITY OF PEPTIDE –OLIGONUCLEOTIDE CONJUGATES

Despite the conceptual advantages of peptide-oligonucleotide hybrids, the analysis of the utility of these conjugates in biological systems is still in its infancy. Most work has concentrated on peptides designed to increase generalised cellular uptake (the so-called protein transduction domain or membrane-penetrating peptides) or confer nuclear delivery. These can be broken down into three broad groups: (i) highly basic peptide sequences that have been identified as the principle protein transduction domains of proteins such as HIV-1 Tat and Antennapedia (ii) hydrophobic signal peptide-like and (iii) nuclear localization sequences (NLS). A summary of peptides (and other carrier mechanisms) used to conjugate to antisense oligonucleotides (or other cargoes) is presented in Tables **2** and **3**.

The basic peptide sequences include the Tat-derived peptide, GRKKRRQRRRPPQT, the Antennapedia-derived peptide, RQIKIWFQNRRMKWKKGGC and further derivatives of both. Tat(43-60), MLGISYGRKKRRQRRRPPQT, when complexed with DNA, has been shown to facilitate delivery of large fragments of DNA into a variety of cell lines, even in the presence of serum [45]. Furthermore, Tat(48-60) is able to successfully translocate a 29 kDa protein, carbonic anhydrase, across the plasma membrane with consequent nuclear and cytoplasmic localisation [46], suggesting that these sequences should enhance both cellular uptake and nuclear accumulation of peptide-oligonucleotide hybrids (the latter being important for antisense molecules acting *via* activation of RNase H).

The peptides, penetratin (Ant(43-58)) and its retro-inverso form, have been used to deliver antisense PNA to cells. Conjugates of Ant(43-58), but not truncated analogues, and a PNA linked by a triglycine spacer were readily taken up by cells without signs of toxicity, but there was no nuclear localization suggesting that a further modification is required to confer nuclear delivery [47]. A retro-inverso form of the antennapedia homeodomain peptide (KKWKMRRNQFWVKVQR) coupled to a PNA induced rapid energy-independent uptake into neurons when compared to an unconjugated PNA [48]. However, although the uptake process was faster, there was no improvement of biological activity and cytotoxicity was evident [48]. The addition of positively charged lysine residues ($(Lys)_4$) on to the end of a PNA oligomer markedly enhanced the cell-penetrating abilities of the oligonucleotide and thus increased nuclear accumulation [49].

Table 2. Studies of Biological Activity for Peptide-Oligonucleotide Conjugates

Peptide	ON	Chemistry	Result	Reference
A. *In vitro*				
(i) Ant(43-58)-(GGC) (ii) Tat(49-60)-(C):	20 mer PS, antiMDR to inhibit p-glycoprotein expression	Joined by disulfide bond	Nuclear accumulation; some endosomal trapping. Conjugate accumulated in cells better than free ON (but not as well as with cationic lipid carrier). Improved antisense activity observed in the presence of serum.	[51]
Ant(43-58)	PNA	Either peptide or PNA as C-terminal domain	Both orientations localized within cells but further modification may be required for efficient nuclear delivery. Presence of peptide did not interfere with hybridisation. No uptake was observed for truncated (minus "RQI") derivatives.	[47]
(i) kFGF (ii) kFGF + (NFκB) NLS	15 or 20mer PS, anti-luciferase	Joined by disulfide bond between 3' and either C or N terminus	Peptide-ON conjugates stable. Efficient intracellular penetration but no antisense activity without cationic lipid. Endosomal trapping in absence of lipid carrier thus preventing antisense activity.	[41]
caiman crocodylus + NLS (SV40)	26mer PS, anti-βCOM	Covalent bond; 5' end to C terminus	Conjugate targets 90% of cells within 5 min. Seven-fold reduction in βsubunit activity (conjugates incubated with cells in absence of serum).	[42]
$(KFF)_3K$	PNA 9-12 mer, anti Acp protein	Covalent bond to PNA N terminus	Conjugate carried into bacteria with no toxicity to HeLa cell where PNA was bactericidal. Antisense activity was a result of improved cell permeabiliz-ation, particularly the outer membrane. Conjugate linker affected antisense activity; maleimide-coupled PNA conjugate was 10-fold less potent than ethylene-glycol linked PNA.	[44, 50]
NLS (SV40)	PNA 17mer anti *c-myc*	Covalent bond at N terminus	Predominantly nuclear localization; PNA alone predominantly cytoplasmic localization. Downregulation of c-myc expression with conjugate only.	[65]
KKWKMRRNQFWVK VQR; retro-inverso form of Ant(43-58)	PNA, 16mer anti-prepro oxytocin		Both conjugate and free PNA internalized (nucleus and neurites) and reduced mRNA. Penetration was increased for the conjugate, but there was no evidence of improved activity.	[48]
(i) Transportan (ii) Ant(43-58)	PNA, 21mer to galanin receptor	Disulfide linked	Conjugate showed moderate accumulation in nucleus whereas PNA alone was found at plasma membrane and in endosomes. Conjugate blocked galanin expression, with an 80-90% reduction in galanin binding; PNA alone was without effect.	[52]
$(Lys)_4$	18mer PNA	Peptide linked to C terminus of PNA	Lysine tail increased the concentration of ON in the cell rather than the number of cells transfected; cellular penetration enhanced.	[49]
JB9	PNA, 12 mer anti-human IGF1 receptor	N terminus of peptide at C terminus of PNA $(Gly)_4$ spacer	High uptake only in cells overexpressing IGF receptor; targeted delivery achieved. Conjugates associated with intracellular structures including the nucleus with no cytotoxicity in either cell line and the analogue was stable in serum. Antisense activity not examined, however, the presence of the peptide did not affect hybridisation.	[39]
B. In vivo				
Ant(43-58)	PNA, 21mer to galanin receptor	Disulfide linked	Intrathecal administration (100 μM) reduced galanin binding (40%) in the dorsal horn of the rat spinal cord.	[52]
C. Other				
(i) gp41b (ii) gp41c	Phosphoramidites: 20mer HIV; 27mer to *rev* mRNA ; 20mer to HIV *tar* mRNA	Covalent link; 3' to N terminus	Thermal denaturation assessed for gp41b conjugate only; hybridisation efficiency maintained with the peptide at 3' terminus (Tm= 73.2 ± 0.1°C), compared with unmodified ODN (Tm= 72.1 ± 0.5 °C).	[22]

(Table 2) contd.....

Peptide	ON	Chemistry	Result	Reference
(i) PTSQSRG-DPTGPKE. (ii) Sar-Leu-Gly-Ile-Gly. (iii) ALPPLE-RLTL. (iv) GALFLG-FLGAAGSTMGAWSQP KSKRKV.	15mer	Peptide conjugated as N-terminal thioester to ON functionalized with cysteine at the 5' end	Successful conjugations achieved for peptides containing a N terminal Pro, Ala or sarcosine. However, due to cyclisation no conjugation product was obtained for the peptide containing a N-terminal Gly.	[21]
(i) Tat-derived, PTSQSRGDPTGPKE. (ii) Cagp41.	Phosphoramidate	Maleimido-derivatised peptide to a 3' cysteine derivatised ON.	Novel strategy for incorporating a 3' thiol	[11]
(i) NLS (SV40) (ii) NLS (nuc-leoplasmine)	Phosphoramidite, 12mer, anti-HA-*Ras*	Post-synthetic conjugation vs stepwise conjugation	Greater stability of conjugates as evidenced by higher melting temperatures. Similar yields and purity obtained with both approaches. Conjugates had higher affinity for DNA but less affinity for RNA.	[10]
(i) α-helical peptide (ii) Fc receptor binding peptide	Phosphoramidate, 20mer to HIV-1 or 21mer rat αfetoprotein	N-terminal maleimido to 5'thiol	High yield of peptide-oligonucleotide conjugate	[9]
Octreotate	PS, 20mer, anti-protooncogene *bcl-2*	Covalent, N terminal maleimido to 5' thiol	SSTR-mediated delivery. Hybridization affinity maintained with the conjugate. Specific binding with nanomolar affinity.	[13]

α-helical peptide: LARLLARLLARL; Ant(43-58)=penetratin: RQIKIWFQNRRMKWKK; ASGP = Asialoglycoprotein; Cagp41: DRVIEVVQGAYRAIRNIPRRIRQG; *caiman crocodylus* signal peptide + NLS (SV40): MGLGLHLLVLAAALQGAKKKRKV; Fc receptor binding peptide: TQPREEQYNSTFRV; gp41b: AVGAIGALFLGFLGAAG; gp41c: ALFLGFLGAAG; *Kaposi* fibroblast growth factor (kFGF): AAVALLPAVLLALLAPC; kFGF + (NFκB) NLS: AAVALLPAVLLALLAPVQRKRQKLMPC; JB9: D-CSKS; D-analogue of insulin-like growth factor (IGF); MAP: KLALKLALKALKAALKLA; NLS= nuclear localization sequence; NLS (nucleoplasmine) = QAKKKKLDK; NLS (SV40)= PKKKRKV; ON = oligonucleotide; PNA = peptide nucleic acid PO = phosphodiester; PS = phosphorothioate; SSTR = somatostatin receptor; SynB1: RGGRLSYSRRRFSTSTGR; Tat(49-60): RKKRRQRRRPPQ; Tat(48-58): GRKKRRQRRRP; Tat(48-60): GRKKRRQRRRPPQ; Tat(47-57): YGRKKKRRQRRR; Transportan: GWTLNSAGYLLGKINLKALAALAKKIL

PNAs have also been investigated for their antibacterial effects. Improved bacterial membrane permeation of PNAs has been achieved with the covalent attachment of the peptide KFFKFFKFFK [44, 50]. Moreover, the linker attachment between PNA and peptide affected antisense activity; the maleimide-coupled PNA conjugate being 10-fold less potent than ethylene-glycol linked PNA. Thus optimization of the linker strategy and improving the peptide carrier could further improve antisense activity [50]. In this instance, passage across the outer membrane was found to be the rate-limiting step [44].

Not all peptide-oligonucleotide conjugates have been successful in translocating their cargo across the plasma membrane to exert an antisense effect. It was shown using several different cell lines that conjugation of the membrane-permeable motif from the hydrophobic region of a signal peptide sequence from *Kaposi* fibroblast growth factor (kFGF), with or without a NLS to a phosphorothioate antisense oligonucleotide, resulted in little or no antisense effect without the aid of a cationic lipid [41]. Even though it was thought that the peptide should assist the oligonucleotide in passing through the cell membrane and reaching the nucleus, localisation of intracellular distribution using fluorescein showed trapping in endosomes. It was therefore concluded that the peptide-oligonucleotide conjugate must also contain an additional feature to avoid endosomal trapping. Thus even though the peptide itself contains membrane translocating and nuclear localisation properties, this does not necessarily result in a reliable delivery system that works well with a number of different cell types [41]. Furthermore, conjugation of a maleimido-derived peptide of the NLS from Simian Virus 40 (SV40) large T antigen to a 5' thiol oligonucleotide enhanced the affinity of the oligonucleotide to complementary DNA, but lowered the affinity (approximately 10-fold) for the target RNA demonstrating that peptide conjugation can, under some conditions, actually reduce binding of oligonucleotides to target RNA [10].

The advent of DNA microarray technology has the potential to improve the screening of antisense oligonucleotides for antisense therapy, or indeed highlight the caution required in data interpretation. Conjugates of Ant(43-58) and an antisense oligonucleotide linked by a disulfide bond have been analysed on a DNA array of 2059 genes. While a reduction (2-3-fold) in the target multidrug resistance gene was observed, there was a concomitant affect on a total of 2% of the other genes as well. Specifically, thirteen other genes were reduced by the conjugate, ten genes were either reduced or increased by the conjugate with the mismatch oligonucleotide and four genes were either reduced or increased by the peptide alone. Furthermore, six other genes were affected by both the antisense and mismatch oligonucleotide conjugates and four genes were increased by all three (the antisense and mismatch conjugates and the peptide alone).

COMPARISON OF PEPTIDES FOR DELIVERY

Few studies simultaneously compare the delivery capabilities of different cell-penetrating peptides, thus making it difficult to determine which peptides confer greatest efficiency for delivery of antisense molecules. Astriab-Fischer *et al.* [51] compared the delivery of a 20 nucleotide phosphorothioate oligonucleotide directed

towards p-glycoprotein that was conjugated to either Tat-(RKKRRQRRRPPQC) or Ant-derived (RQIKIWFQNRRMKWKKGGC) peptides. Expression of p-glycoprotein was successfully knocked down at submicromolar concentrations with peptide–oligonucleotide conjugates whereas, oligonucleotide alone was relatively ineffective. Both conjugates were found to accumulate in cells to a greater degree than 'naked' oligonucleotides but to a lesser degree than those delivered using a cationic lipid. Of particular note was the fact that these peptide-oligonucleotide conjugates also functioned in the presence of serum—this result was in striking contrast to other approaches for the intracellular delivery of nucleic acids [51]. While nuclear accumulation was observed, punctate cytoplasmic fluorescence was also detected indicative of endosomal trapping [51]. Intracellular delivery appeared to be similar for both peptides.

Although exhibiting differing intracellular distribution, a similar antisense activity was observed for transportan (GWTLNSAGYLLGKINLKALAALAKKIL) and Ant(43-58) (RQIKIWFQNRRMKWKK) when linked to a PNA directed against the galanin receptor gene [52]. Conjugation was *via* a disulfide bond, which, as discussed above, may detach the PNA from the peptide after reduction in the intracellular milieu. In melanoma cells, intracellular distribution was predominantly membranous and nuclear for transportan and Ant(43-58) conjugates respectively, with both retaining a strong antisense effect *in vitro* with 83% and 91% reduction in galanin binding, respectively [52].

A comparative analysis of peptide-mediated oligonucleotide delivery using several structurally-modified peptides provided evidence that amphipathicity and α-helicity are not essential for cell penetration of peptide-oligonucleotide conjugates [53]. Amino acid sequences were altered from an original sequence to reduce or eliminate α-helical content and/or amphipathicity and cellular uptake of subsequent peptide-oligonucleotide was determined. Interestingly none of the alterations substantially reduced oligonucleotide delivery which was consistently an order of magnitude greater than oligonucleotide alone [53]. The results suggest that the enhanced cellular delivery of peptide-conjugated oligonucleotides in this case may be related to increased affinity for target, reduced oligonucleotide efflux or altered interaction with oligonucleotide binding proteins rather than improved membrane translocation.

MEMBRANE-PENETRATING PEPTIDES AS POTENTIAL FACILITATORS OF OLIGONUCLEOTIDE DELIVERY

Peptides do not necessarily need to be conjugated to antisense oligonucleotides in order to be considered as possibilities for use in antisense therapy; lessons can be learned from all forms of peptide conjugates. With this in mind, the peptides outlined below may have utility as antisense oligonucleotide delivery agents.

The primary amphipathic peptide, MGLGLHLLVLA-AALQGAKKKRKV, a combination of the signal sequence from caiman crocodylus Ig(v) and the nuclear localization sequence of SV40 large T-antigen, has been conjugated to the antitumour drug porphyrin through covalent linkage at the C-terminal cysteamide. In isolated cells, a 5 minute incubation resulted in 80% uptake of the compound and localization was predominantly, although not exclusively, nuclear; without peptide, uptake was confined to approximately 3% of cells, and localization was cytoplasmic [54].

Another peptide worth considering is that derived from the homeodomain of the rat transcription factor Islet-1 (pIs1; RVIRVWFQNKRCKDKK). This peptide is able to transport a large cargo (avidin) as a complex into the cell in a nonendocytotic manner with an intracellular localization similar to that of penetratin. One advantageous feature is that it contains a native cysteine residue, which may be useful for coupling reactions of cargoes [55]. Given the similarity to penetratin (whose utility is discussed above), it may be of interest to study the carrier properties of this peptide when conjugated to an antisense oligonucleotide.

Using a covalently attached pentapeptide as cargo, Hallbrink *et al.* [56] compared amphipathic helical peptides, transportan (GWTLNSAGYLLGKINLKALAALAKKIL) (where lysine was the main contributor to the positive charge) and a model amphipathic peptide (MAP; KLALKLALKALKAALKLA) and each of the arginine-rich peptides, Tat(48-60) (GRKKRRQRRRPQ) and Ant(43-58) (RQIKIWFQNRRMKWKK). While uptake and cargo delivery, from fastest to slowest was found to be MAP, transportan, Tat(48-60) then Ant(43-58); both MAP and transportan caused greater membrane damage compared to Tat(48-60) and Ant(43-58). Given the membrane damage incurred with MAP and transportan, these data indicate that Tat- and Ant-derived peptides may have greater promise as delivery agents.

Membrane penetrating peptides derived from viruses are not the only means of penetrating the cell. The use of stretches of arginine residues has also been investigated with Arg9 peptide carriers being as efficient as Tat(48-60) in translocating a 29 kDa protein across the plasma membrane leading to both nuclear and cytoplasmic localisation [46]. Covalent coupling of a peptide derived from anti-DNA monoclonal antibodies, VAYISRGGVSTYYSDTVKGRFT-RQKYNKRA to protein macromolecules (e.g., horseradish peroxidase) through a cysteine residue allowed effective translocation across plasma and nuclear membranes, possibly through the involvement of an α-helix.

In Vivo Studies

Carrier-peptide mediated delivery is a receptor- and transporter-independent pathway, and unfortunately at this point in time few *in vivo* reports exist describing the utility of administering peptide-oligonucleotide conjugates to whole animals. However, to date, although not always conjugated to antisense molecules, the HIV Tat-derived peptide (YGRKKRRQRRR), penetratin or Ant(43-58) (RQIKIWFQNRRMKWKK) and the linear peptide SynB1 (RGGRLSYSRRRFSTSTGR), all appear promising.

For example, intrathecal administration (150μM three times at 12 h intervals) of the Ant(43-58) peptide conjugated to a PNA directed to the galanin receptor resulted in a down-regulation of galanin receptors in the dorsal horn of the rat spinal cord with no apparent signs of toxicity [52].

Table 3. Delivery of Oligonucleotides with Nonpeptide Delivery Agents and Peptides Used for Delivery of Non-Oligonucleotide Cargoes

"Carrier"-oligonucleotide conjugate				
Carrier	**Cargo**	**Chemistry**	**Result**	**Reference**
A. In vitro				
Lactose (ligand for ASGP receptor)	PNA, 13mer anti-telomerase	Amide-linked Disulfide bond	PNAs selectively taken up into cells containing the ASGP receptor. Improved antisense activity relative to PNA alone, but 50-fold less efficient than delivery with a cationic lipid. Compartmentalized on entry hindering efficient release into cytoplasm.	[63]
ASGP	Phosphoroamidite, 15mer, anti-gp 130	Disulfide linked; six ONs per ASGP	Conjugates inhibited up-regulation; unconjugated ON showed no inhibition. Using covalent chemistry rather than complex reduced the amount of ASGP required.	[16]
Cholesterol	25mer PO	SH-derivatised ON linked to thiol-derivatised cholesterol	Cell binding and internalisation are modified depending on where cholesterol moiety is conjugated; linking at positions 3 and 7 of cholesterol results in fast uptake, position 22 was slow and inefficient.	[66]
B. In vivo				
OX-26	PNA, 16mer anti-luciferase, radioiodinated		Conjugate showed increased metabolic stability. Following intravenous administration to rats there was enhanced brain and liver uptake with reduced kidney and heart uptake. Able to image gene expression in rat with experimental brain tumour.	[67]
OX-26-SA	PNA 18 mer		Brain penetration of PNA negligible however conjugation to OX-26 SA increased uptake in the order of 28-fold. This was approx 0.1% of injected dose.	[61]
L_3G_4	Miscellaneous PO		Anaesthetized rat, intravenous. Monitor hepatic uptake	[62]
Cholesterol	PS; ISIS 9388	3' conjugation	Cholesterol conjugation resulted in high accumulation in liver of rat 3h after intravenous administration (approx. 2.5 times that of unconjugated).	[68]
C. Other				
(i) Folic acid (ii) Retinoic acid (iii) Arachadonic acid	PS	Attached at 5' end	Stable duplexes formed with target	[59]
Peptide-"cargo" conjugate				
A. In vitro				
(i) Ant(43-58) (ii) Tat(48-60): (iii) MAP (iv) Transportan	Pentapeptide LKANL	Disulfide bond	Higher delivery efficiency with MAP and transportan than with penetratin and Tat(48-60) but MAP and transportan induced membrane leakage, penetratin or Tat(48-60) did not.	[56]
Tat(47-57):	βgalactosidase	Fusion	Rapidly transduced into cells, maximal intracellular concentration within 15 min. Enzymic activity peaked after 2h.	[69]
Tat(48-58)	Biotin/streptavidin	6-aminohexanoic acid linker	Following intravenous administration of the conjugate there was an increase in membrane permeation with a parallel decrease in plasma area under the curve with only a modest affect on organ uptake (as % injected dose).	[70]
Tat(48-60)-C and equally efficient peptides: (i)HIV-1 Rev(34-50) (ii)FHV coat (35-49) (iii)R_9- Tat	Carbonic anhydrase (29kDa)	Cys of peptide reacted with maleimido group on protein	Accumulation in cytosol and nucleus; without peptide distribution was limited to parts of the cytosol. Optimal number of arginine residues for efficient translocation is 8	[46]
MGLGLHLLVLAA ALQGAKKKRKV (signal peptide + NLS)	Porphyrin (potential antitumour drug)	Covalent	80% uptake within 5 min with localization mainly nuclear; some cytoplasmic. 100-fold increase in porphyrin activity as a conjugate.	[54]
VAYISRGGVSTYY SDTVKGRFTRQKY NKRA (derived from a monoclonal Ab.	Horesradish peroxidase	Covalent	Cytoplasmic or nuclear labelling depending on the cell line.	[58]
B. In vivo				
(i) D-Ant(43-58) (ii) SynB1	Doxorubicin		Following intravenous administration to mice there was a increase in brain uptake of the doxorubicin conjugate with a concomitant reduction in the amount found in heart.	[58]
Tat(47-57):	βgalactosidase	Fusion	Following intraperitoneal administration to mice, fusion molecule was transduced into blood, muscle and splenic cells. Strong βgalactosidase activity was detected in liver, kidney lung and heart; weak activity in spleen. Enzyme activity also found in all regions of the brain, including cell bodies.	[69]

Ant(43-58)=penetratin: RQIKIWFQNRRMKWKK; ASGP = Asialoglycoprotein; FHV coat(35-49): RRRRNRTRRNRRRVR-GC; HIV-1 Rev(34-50): TRQARRNRRRRWRERQR-GC; L3G4 = Ligand for ASGP receptor = N^2-[N^2-(N^2, N^6-bis{*N*-[*p*-(β-D-galactopyranosyloxy)-anilino]thiocarbamyl}-L-lysyl)-N^6-(*N*-{*p*-[β-D-galactopyranosyloxy]-anilino}thiocarbamyl)-L-lysyl]–N^6-[*N*-(*p*-{β-D-galactopyranosyloxy}-anilino)thiocarbamy]}-L-lysine; MAP: KLALKLALKALKAALKLA; NLS= nuclear localization sequence; ON = oligonucleotide; OX-26: Monoclonal antibody to the rat transferrin receptor, OX-26-SA: streptavidin conjugated to OX-26; PNA = peptide nucleic acid PO = phosphodiester; PS = phosphorothioate; R_9 Tat: GRRRRRRRRRPPQ-C; SynB1: RGGRLSYSRRRFSTSTGR; Tat(47-57): YGRKKKRRQRRR; Tat(48-58): GRKKRRQRRRP; Tat(48-60): GRKKRRQRRRPPQ; Transportan: GWTLNSAGYLLGKINLKALAALAKKIL

There is a paucity of literature with antisense molecules, however, there are a few additional studies investigating delivery of other membrane impermeable molecules conjugated to peptides and these may be instructive on potential behaviour of these peptides as antisense delivery agents. A Tat(48-59) (GRKKRRQRRRAP)-β-galactosidase fusion protein demonstrates an intracellular nuclear accumulation, *in vitro* [57]. Extension of this work to mice using intraperitoneal administration of a similar fusion protein, fluorescently-labelled Tat(YGRKKRRQRRR)-β-galactosidase demonstrated significant distribution to blood, splenic cells and muscle. In contrast, control β-galactosidase alone could not be detected. Furthermore, strong enzyme activity was detected in liver, kidney, lung and heart at 4 and 8 h, whereas the activity of the unconjugated β-galactosidase could only be reconciled with lymphatic uptake from peritoneum. Of particular interest was the ability of this conjugate, but not β-galactosidase alone, to penetrate the central nervous system such that enzyme activity was detected in cell bodies. It is likely that the protein is able to enter the nucleus by the embedded NLS in the Tat protein transduction domain.

Two other peptide vectors have been found to penetrate the blood brain barrier; D-penetratin (RQIKIWFQNRR-MKWKK) and SynB1 (RGGRLSYSRRRFSTSTGR). Again these peptides were not conjugated to antisense oligonucleotides, but rather the antineoplastic agent doxo-rubicin [58]. Following intravenous administration to mice, an improvement in the brain distribution was noted for the conjugate as compared to unconjugated drug. Furthermore, there was a concomitant reduction in distribution to heart and lung and a slight decrease in liver and kidney [58].

TARGETED DELIVERY OF OLIGONUCLEOTIDES

The ability of certain peptides to penetrate membranes is not the only cellular mechanism that can be exploited in order to deliver oligonucleotides to cells; more targeted delivery can be achieved by exploiting specific receptor-mediated mechanisms. Classic drug-based therapies predominantly target cell-surface receptors. Such therapies utilise the specific distribution of receptors as a mechanism for obtaining specificity. Combination of peptide receptor ligands with antisense oligonucleotides provides a theoretical increase in specificity as well as a dramatic increase in the scope of activity of the "drug". Receptor-based therapy is by its very nature limited to the scope of actions mediated by the targeted receptor. However the combination of a cell-targeting peptide, together with receptor-mediated delivery has the potential to dramatically increase the efficiency of antisense targeting.

It is known that there is an overexpression of somatostatin receptors (SSTRs) on tumours, thus these membrane-associated receptors are a viable means of selective delivery of agents to tumours/tumour cells. Mier *et al.* [13] designed peptide-oligonucleotide conjugates to be targeted to the SSTR on the plasma membrane. The N-terminal maleimido-derivatized peptide, tyr3-octreotate, the carboxylic acid derivative of octreotide, was conjugated to a 5' thiol 20 nucleotide phosphorothioate oligonucleotide. To date, biological assessment of this conjugate has been restricted to a receptor binding assay in rat cortex membranes that predominantly express the SSTR-2. IC_{50} of the conjugates were in the nanomolar range demonstrating high affinity for SSTRs. While these data hold promise for the selective delivery of antisense oligonucleotides to tumours, to date, the *in vivo* biodistribution of these conjugates is not available.

Conjugates of antisense oligonucleotides and folic acid, retinoic acid, arachadonic acid or methoxypoly(ethylene glycol) propionic acid have also been assessed [59]. The basis of these combinations is that many tumours often over express receptors for certain growth factors, vitamins and hormones thus antisense oligonucleotides will be taken up by a receptor-mediated endocytotic mechanism. Although no *in vitro* cell assays were performed, oligonucleotide conjugates were said to form stable duplexes with the target mRNA.

To enhance penetration across the blood-brain barrier, phosphorothioate or phosphodiester oligonucleotides or PNAs have been conjugated to a OX-26-streptavidin vector, where OX-26 is a monoclonal antibody to the rat transferrin receptor [60]. The transferrin receptor is located both at the blood-brain barrier and in the liver thus antisense oligonucleotides would be directed to both these locations rather than being cleared by the kidney. While OX-26 delivery of PNAs across the blood brain barrier of rats was achieved with this approach, species specific antibodies may need to be developed and the consequence of presence of the antibody on uptake of the antibody-oligonucleotide conjugate into brain cells needs to be addressed [61].

Targeted delivery of antisense oligonucleotides to the liver can be achieved by derivatizing the oligonucleotide with a galactose-based ligand for the asialoglycoprotein receptor allowing the oligonucleotide to accumulate in parenchymal liver cells [62]. Using this method, *in vivo* hepatic uptake following intravenous injection was 77% of administered dose compared with an uptake of 19% with unconjugated oligonucleotide. PNAs have also been delivered *in vitro* using lactose, a ligand for the asialoglycoprotein receptor. PNA-lactose conjugates were joined with several lysines, with or without an additional disulfide bond [63]. While no antisense activity was observed for the unconjugated PNA (directed to telomerase), both conjugates inhibited telomerase activity with a lower IC_{50} and there was an increased cytoplasmic and nuclear localization reported for the conjugate containing the disulfide bond. However, the inhibition observed with the PNA-conjugates were 50-fold less than earlier experiments using a cationic lipid as the delivery agent [63]. This latter observation may be due to compartmentalization of the conjugate with punctate localization being indicative of endosomal trapping, thus as with other conjugates, a mechanism for endosomal escape is required.

PNAs have also been conjugated to an analogue of the insulin-like growth factor 1 (IGF1) (Cys-Ser-Lys-Cys) in order to direct delivery in a cell- and tissue-specific manner *via* the cell surface receptor for IGF1. In this case a four glycine spacer was added between the PNA and the peptide. This conjugate was resistant to proteases and nucleases and accumulated within intracellular structures, including nuclei following internalization, with no evidence of toxicity [39].

CONCLUDING REMARKS

Pharmacological applications of oligonucleotides have been hindered by the inability to effectively deliver these compounds to their sites of action within cells. Many studies have been conducted on the preparation of peptide–oligonucleotide conjugates with the intent of improving intracellular delivery. For this purpose, the two most common peptides used are a sequence from HIV Tat, RKKRRQRRRPPQC, and a sequence from the homeodomain of antennapedia, (RQIKIWFQNRRMKWKK-GGC). However as the chemistry of peptide-oligonucleotide conjugation has advanced to a point where we can consider attachment at either the amino or carboxyl terminus of peptides and to either the 3' or 5' ends of oligonucleotides, we thus have the capacity to assemble more complex hybrids. For instance, incorporation of a receptor-specific cell targeting peptide, an antisense oligonucleotide and a generalised cell delivery peptide. Whether such complex hybrids would have an increased utility remains to be explored as indeed are issues relating to the impact on oligonucleotide activity and thus optimisation of this component. For example such modifications may lead to total abolition of activity, change in optimal oligonucleotide length or no effect at all. Similarly, one could envision incorporation of two antisense oligonucleotides. Indeed the chemistry has been investigated for this using alanine (n) as the peptide [34].

The investigation of membrane penetrating peptides as delivery vectors for antisense oligonucleotides is still in its infancy. Relatively few sequences have been subject to detailed and systematic studies for mechanism of action and efficiency of target knockdown *in vivo*. This could be enhanced by the use of protocols such as phage display technology to screen for peptide motifs with greater efficiency at entering particular target cells [64]. Likewise, this review demonstrates that there are currently a large number of methods available to achieve linkage of oligonucleotides to vector peptides but no protocol has yet become routine for this purpose. Detailed comparative studies on linkage chemistries and subsequent biological activity of conjugates in a standardized format is urgently required before peptide-oligonucleotide conjugates can become potent tools in therapeutic delivery of antisense molecules.

ABBREVIATIONS

2-O-MOE = 2'-O-(2-methoxyethyl)

AOQ = 4-(2-aminooxyethoxy)-2-(ethylureido)quinoline

ASGP = Asialoglycoprotein

CPG = Controlled pore glass

EOQ = 4-ethoxy-2-(ethylureido)quinoline

HIV = Human immunodeficiency virus

IC_{50} = Inhibitory concentration 50%

IGF1 = Insulin-like growth factor 1

kFGF = *Kaposi* fibroblast growth factor

MAP = Model amphipathic peptide

Mtt = Methyltrityl

NLS = Nuclear localization sequence

Orn = Ornithine

pIs1 = Rat transcription factor Islet-1

PNA = Peptide nucleic acid

SSTR = Somatostatin receptor

SV40 = Simian virus 40

tBu = Tert-Butyl

TFA = Trifluoroacetic acid

THF = Tetrahydrofuran

Tat = Trans-acting transcriptional activator

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