CMLS, Cell. Mol. Life Sci. 60 (2003) 844–853
1420-682X/03/050844-10
DOI 10.1007/s00018-003-3044-5

CMLS Cellular and Molecular Life Sciences

Peptide-mediated cellular delivery of antisense oligonucleotides and their analogues

Michael J. Gait

Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH (United Kingdom), Fax: +44 1223 402070, e-mail: mgait@mrc-lmb.cam.ac.uk

Abstract. Improving the delivery of synthetic oligonucleotides and their analogues into cells is an important goal in the full development of antisense technology for control of gene expression in cell culture and in vivo. This review describes the harnessing of certain peptides, either as noncovalent complexes or as covalent conjugates, to enhance the delivery of antisense oligonucleotides into cells and/or to affect their cell localization. Phosphodiester and phosphorothioate oligonucleotides are included as well as peptide nucleic acids (PNAs), analogues of oligonucleotides where the negatively charged phosphate backbone is replaced by a neutral amide linkage. This review contains a critical evaluation of claims for certain peptide-oligonucleotide conjugates to translocate into cultured cells by a non-energy-dependent nonendosomal route. In addition, the available evidence for the utility of stable versus nonstable linkages between peptide and oligonucleotide or PNA is discussed.

Key words. Antisense; conjugates; oligonucleotides; peptides; PNA.

Introduction

In a recent review article on nucleic acid therapeutics, Opalinska and Gewirtz state: 'Another problem in this field is the limited ability to deliver nucleic acids into cells and have them reach their target' [1]. These authors refer primarily to synthetic antisense oligonucleotides and their use in the clinic as drugs, particularly as anticancer and antiviral agents [2, 3]. They argue cogently that advances in antisense delivery are essential if effective diagnoses and treatments are to be attained [1]. However, the needs for improved delivery extend also to the widespread application in functional genomics by targeted antisense knockdown of gene expression in cultured cells (reviewed in [4]), and it is to this end that the vast majority of efforts have been directed. Transfection of DNA and oligonucleotides can often be achieved for common laboratory cell lines in culture (e. g. cancer cells) through use of cationic lipids [5]. But this is far from being universally true, especially for primary cells. Much recent interest has been aroused by the suggestion that certain peptides can dramatically enhance cell penetration and, in some cases, cellular targeting of antisense agents. This review focuses on recent literature on the subject of their peptide-mediated cell delivery, notably into cultured eukaryotic cells. This relatively new field has unearthed unexpected problems, particularly in the interpretation of results, rather than so far having provided definite solutions.

A major difficulty is that the subject cuts across traditional scientific boundaries. Rigorous experimentation in chemistry, molecular biology and cell biology is required, and expertise in all three disciplines is not often available in a single laboratory. Thus, established general principles are sparse. For example, cell uptake, cell activity and cell toxicity of peptide/oligonucleotide complexes or conjugates have rarely been measured simultaneously. Further in the case of conjugates, few studies have utilized more than a small number of different chemical constructs, and therefore structure-function relationships are generally lacking, even within a single test system. In addition, controversies over the use of certain cell fixation agents in microscopy have clouded the interpretation of cell localization images, especially for earlier literature. In this review, we have not aimed at comprehensiveness, but instead selected key recent references to point readers towards important issues in this challenging new field. More in-depth accounts of a range of cell penetrating peptides (CPPs) can be found in a recent book [6], and other reviews of uses of cell delivery peptides have been published [7, 8].

Getting to grips with parameters

So far no 'magic bullet' CPP has emerged that can carry antisense oligonucleotides and analogues universally into a wide range of cultured cell lines, including primary cells. Instead, much of the literature has focused on the potential for certain peptides to enhance uptake for a particular class of oligonucleotide analogue into certain cell lines. Some peptides has been investigated for their potential to localize oligonucleotides into particular cell compartments (e.g. nucleus or cytosol). 'Cargoes' are generally divided into three groups: (i) double-stranded DNAs or plasmids (not discussed here but reviewed in [9]), (ii) short unstructured oligonucleotides and analogues containing formal negative charges (e.g. phosphodiesters or phosphorothioates) and (iii) charge-neutral oligonucleotide analogues, notably peptide nucleic acids (PNAs). From the few studies involving more than one cargo group, it is clear that cell uptake is not independent of cargo type, size or chemistry, which are all integrally important parameters. Even within a cargo class, little knowledge exists on how cell delivery is modulated by cargo composition or chemistry. This is a serious issue, since antisense oligonucleotide types and lengths vary extremely widely. Other important parameters include whether the peptide is covalently attached or merely complexed. In the former case, there are further, mostly unstudied parameters of orientations of the peptide and oligonucleotide as well as types of linkage. In the latter case, the stoichiometry of complexation and physical formulation may affect cell uptake. Finally, there are questions of cell type, handling and growth conditions, as well as variations of technique in activity assay determinations, cell fixation and imaging. Such a number and complexity of parameters has led to an imbalance towards qualitative rather than desirable quantitative data.

Mechanistic controversies in cellular uptake

Antisense oligonucleotides of the negatively charged variety are thought to be taken up by fluid phase or adsorptive endocytosis. This process is energy dependent and saturable. Thus, uptake of naked phosphodiester or phosphorothioate oligonucleotides is usually inefficient in most cell lines in culture, hence the need for transfection reagents either as additives or as conjugates. Considerable interest has been generated from the discovery that certain peptides apparently translocate across cell membranes by a mechanism that does not involve receptor-mediated endocytosis. First and foremost amongst such peptides are residues 43–58 of the third helix of *Antennapedia* homeodomain (known as Penetratin) [10, 11] and the highly Arg/Lys-rich region of the human immunodeficiency virus (HIV)-1 Tat protein (Tat peptide) [12]. In the case of Tat peptide in particular, a number of different materials have been conjugated and delivered into cells in culture. This includes proteins [13, 14], liposomes [15] and λ phage-packaged DNA [16]. Tat peptide has been used in vivo to deliver β-galactosidase as a Tat fusion into all tissues in mice [17], metal chelates for medical imaging [18] and superparamagnetic nanoparticles for tracking and recovery of progenitor cells [19]. For protein delivery in vivo, its use may be limited by rapid plasma clearance [20]. Recently, it has been shown that the mechanisms of Tat protein and Tat peptide uptake may be different and that in contrast to Tat protein, uptake of Tat peptide does not involve binding to heparin sulfate proteoglycans and endocytosis [21]. A slightly longer synthetic peptide called transportan (a hybrid of a section of the neuropeptide galanin and the wasp venom peptide mastoparan) has recently joined the other two in popularity, and has been used in particular for PNA delivery [22]. These three CPPs, usually fluorescently labelled, and a number of their derivatives have shown remarkable cell uptake properties (all reviewed in detail in [6]) by a mechanism that appears, for the free peptides at least, to rule out endocytosis. These peptides do not generally show cell toxicity at concentrations of a few micromolar or less, which is in contrast to many cationic lipid preparations. More recently several other peptide types have been investigated for cell import characteristics. Yet controversies linger as to whether, and under what circumstances, such peptides can deliver oligonucleotide cargoes into cells, and if so, what mechanisms of uptake are actually being used.

Peptide complexation of antisense oligonucleotides

A number of peptides or peptide derivatives are cationic and are used to condense or 'package' DNA, in a similar way to encapsulation by cationic lipid, and thus may enhance release of DNA from endocytotic vesicles. For example, a peptide composed of Cys-Trp-Lys_{18} was very efficient for gene transfer into HepG2 cells and COS cells [23]. More recently, a cationic surfactant GS11 composed primarily of a dimeric lysine-rich lipopeptide, when combined with conventional DOPE lipid, was shown to give effective and nontoxic DNA delivery into a range of eukaryotic cell lines [24]. GS11 delivery of oligonucleotide analogues into HeLa cells is also effective [A. Arzumanov and M. J. Gait, unpublished results].

A long cationic amphipathic peptide composed mostly of repeated KALA units was found to bind both DNA and phosphorothioate oligodeoxyribonucleotides and to increase transfer from punctate cytosolic locations into the nucleus of several cell lines, notably CV-1 fibroblasts and cancer cells [25]. This and other similar cationic peptides [26, 27] presumably alter their conformation at slightly

acidic pH and aid endosomal release of DNA. Protamine, a polycationic peptide of ca. 4000 molecular weight, formed nanoparticle complexes with phosphodiester oligonucleotides ('proticles') and delivered them into human lymphoma U-937 cells [28]. The mass ratio and particle size were important parameters to attain both good delivery and anti-c-*myc* antisense activity. Although relatively nontoxic, there were some nonspecific antisense effects observed with the proticles. An anionic peptide derived from the N-terminus of the HA2 subunit of influenza virus hemagglutinin was shown to deliver phosphorothioate oligodeoxynucleotides into adherent as well as nonadherent cells and direct them into the nucleus. But the peptide was rather toxic and could only be used in a narrow concentration range [29].

Of greater fascination are studies of multifunctional peptides that contain two juxtaposed sections designed for DNA complexation, membrane transportation and/or nuclear delivery. A 1:1 complex of a peptide composed of the Kaposi fibroblast growth factor (K-FGF) signal peptide and a 10-mer of poly (L-lysine) with a fluorescently labelled 10-mer phosphorothioate oligonucleotide was found to be taken up efficiently into the nucleus of A-549 adenocarcinoma cells without concomitant toxicity, but no antisense activity studies were reported [30]. A second peptide named MPG, comprised of an HIV-1 gp41 fusion peptide and a sequence derived from the SV40 T-antigen nuclear localization signal (NLS), protected oligodeoxyribonucleotide phosphodiesters and phosphorothioates from nuclease degradation in culture medium, when used in 20–50-fold excess, and delivered them effectively to the nuclei of fibroblast cells in culture [31]. Once again, no antisense activity data were reported. Both of these above peptides were claimed to direct oligonucleotide entry but avoid passage through endosomal vesicles, because uptake was still observed at 4 °C and because punctate cytosolic fluorescence characteristic of endosomal locations was absent. Similar claims for non-energy-dependent cell delivery have been made for a fusion of a peptide derived from membrane-active dermaseptin and a nuclear localization signal from SV40 T antigen or from the HIV-1 Rev-responsive element [32]. Interestingly, nuclear import was blocked at lower temperature, suggesting an energy-dependent nuclear import pathway. So far, nucleic acid delivery with these peptides has not been reported.

A more useful study has been published recently comparing both delivery and antisense activity mediated by a combination peptide consisting of a highly arginine-rich fragment of bull protamine and the NLS from SV40 T antigen. Peptide complexes with 20-mer and 18-mer phosphorothioate oligonucleotides respectively targeted against protein kinase C (PKC)-α and bcl-2 were shown to localize in the cell nucleus and to exert the expected sequence-specific antisense activities [33]. However, in some cell lines (notably PC3 prostate cancer cells) it was necessary to include chloroquine, a lysosomotropic reagent, to obtain delivery and antisense activity. These results suggest that the peptide acts to bind oligonucleotide fragments and that the predominant uptake pathway of the complexes is still endosomal. This is despite strong evidence that arginine-rich peptides themselves enter cells by an energy-dependent mechanism but which does not involve endocytosis [34].

An interesting paper described the use of an integrin receptor-targeted peptide containing an RGD motif arranged as a tetrameric branch from two Lys residues radiating from a zinc-finger domain of HIV nucleocapsid protein. This peptide was shown to improve antisense phosphodiester oligodeoxyribonucleotide activity 50-fold in a human myeloid cell line HL-60 compared with the absence of a delivery system [35]. A dendritic peptide made up of a number of SV40 NLS sequences joined to a sequence of five lysines and branched through other lysines from a Cys-Gly-Tyr spacer ('Loligomer') has also been used to deliver DNA into the nuclei of cells [36], but disappointingly, no antisense delivery studies have yet been reported.

Peptide conjugates of oligonucleotides with phosphodiester or phosphorothioate backbones

In contrast to the use of complexes, covalent conjugates of peptides and oligonucleotides are discrete chemical entities of known stoichiometry. In principle, greater reproducibility of reagent preparation and use should be achievable. This is balanced by the synthetic difficulties hitherto in their production. Excellent reviews of the chemical synthesis of peptide-oligonucleotide conjugates have been published recently [37–39], and peptide conjugation is usefully compared with conjugation of other molecule types in another recent review [40]. Although the current review is not concerned primarily with chemical synthesis, it is important to note that linkages between peptide and oligonucleotide can be of two classes. Stable covalent bonds may be created through, for example, amide, thioether, thiol-maleimide, thiazolidine, oxime or hydrazine linkages. By contrast, a commonly utilized method of conjugation involves formation of a disulfide bond. This is thought to cleave rapidly once within the reducing environment of the cell. It is not yet clear whether use of cleavable bonds offers advantages or may be necessary for certain applications.

Methods of solution-phase conjugation have frequently proved laborious, time-consuming and prone to poor yields, explaining the general lack of structure-function studies. The best hope for the future might be methods involving total solid-phase synthesis of peptide-oligonucleotide conjugates [41–43], although none of these is universally applicable or is sufficiently flexible as yet.

Cellular uptake and antisense activity of peptide-oligonucleotide conjugates

Biological results with antisense oligonucleotides linked to peptides have been remarkably few. One of the first reports was for 15–17-mer antisense oligodeoxyribonucleotides conjugated to poly (L-lysine). Preincubation of such conjugates with L929 cells resulted in a 50-fold better protection against infection by vesicular stomatitis virus (VSV) compared with the unconjugated oligonucleotide [44]. Improved uptake both in speed and quantity into L929 cells was found, and adsorptive endocytosis was suggested as the uptake pathway [45]. However the molecular mass of the poly (L-lysine) was large (14,000 Da). Similarly, a 12-fold increase in protection was obtained against HIV in an acute infection test in MT-4 cells. Here the oligonucleotide conjugate with poly (L-lysine) was directed against the HIV Tat messenger RNA (mRNA) [46]. Unfortunately, the use of poly (L-lysine) is limited by cytotoxicity and complement activation.

Another early study involved attachment of a fusogenic peptide, representing part of the N-terminus of the HA-2 subunit of influenza virus haemagglutinin, to an antisense oligodeoxyribonucleotide either through a disulfide bond or a thioether bond. Such conjugates gave a 5–10-fold increase in anti-HIV activity in a de novo infection assay in CEM-SS lymphocytes [47]. However, the activity was not sequence specific, and the peptide possessed its own antiviral activity. Fluorescently labelled conjugates showed a less punctate cellular distribution than the free oligonucleotide, and no uptake was observed at low temperature, suggesting use of the endosomal delivery pathway. Stable thioether bonds were more effective than disulfide bonds in conjugates of a 25-mer phosphodiester oligodeoxyribonucleotide targeted to HIV-1 gag mRNA to a peptide containing a signal peptide KDEL domain, which is known to target proteins to the endoplasmic reticulum [48]. The thioether linked conjugate, which was 3′ capped to enhance stability, showed 3-fold higher activity than the free oligonucleotide and 10-fold higher activity compared with the 3′-disulfide-linked oligonucleotide in infected human peripheral blood mononuclear cells (PBMCs), which was attributed to the instability of the disulfide bond, leading to 3′-exonuclease degradation of the oligonucleotide during endocytosis. These KDEL-tagged oligodeoxyribonucleotides were shown to traffic to the endoplasmic reticulum and the Golgi, and had a lower efflux from HepG2 cells than unconjugated oligonucleotide [49].

Antisense conjugates of 15-mer oligodeoxyribonucleotides were linked through the 5′ end by disulfide to thiol-modified asialoglycoprotein (ASGP), a protein that targets receptors on hepatocytes, at a ratio of about six oligonucleotides to one protein [50]. The conjugates showed enhanced and sequence-specific antisense inhibition of interleukin (IL)-6-mediated acute phase protein production in HepG2 cells, but no cell uptake or localization studies were reported.

Perhaps the most controversial area of study centres on peptide-oligonucleotide conjugates where the unconjugated peptides are thought to be delivered into cells by a nonendosomal route. Two early antisense reports are particularly intriguing. The 16-residue peptide *Antennapedia* peptide (penetratin) linked by disulfide bond to a 5′-thiol-substituted oligodeoxyribonucleotide showed sequence-specific downregulation of amyloid precursor protein (APP) mRNA at a remarkably low concentration (75 nM) in cultured primary neurones [51]. Fluorescently labelled conjugates accumulated in the cytosol and nucleus of nerve cells by a process that apparently did not require endocytosis. The disulfide bond was believed to be cleaved during cell entry. A second example of enhanced antisense activity was observed in PC-12 neuronal cells through use of penetratin-linked oligonucleotides complementary to the mRNA for Cu/Zn superoxide dismutase [52]. More recent studies have been very revealing. A disulfide-linked conjugate of penetratin to a 20-mer phosphorothioate oligodeoxyribonucleotide was taken up into the nucleus of mouse 3T3 cells stably expressing the *MDR1* gene and blocked expression of P-glycoprotein synthesis at submicromolar concentrations in a sequence-specific manner [53]. The activity was observed also in the presence of serum. Identical results were obtained also with conjugates containing the Tat peptide. Interestingly, microscopy studies showed similar patterns of distribution for the conjugates compared with cationic lipid oligonucleotide delivery, notably both punctate cytosolic as well as more uniform nuclear localization (fig. 1). In a further study by the same authors, Tat or penetratin was coupled by disulfide bond to an 18-mer 2′-*O*-methyl oligoribonucleotide phosphorothioate and shown to localize in HeLa cells both strongly in the nucleus and in large cytosolic vesicles [54]. Uptake was shown to be clearly endocytotic and energy dependent. The conjugated oligonucleotides were shown to have dose-dependent antisense activity in steric block mode by correcting a splicing-defective luciferase gene that was stably integrated into the HeLa cells.

Only one other significant report has been published of successful antisense activity and cell uptake being directed by a peptide conjugate. Two alternative composite peptides (the signal sequence from *Caiman crocodylus* immunoglobulin (Ig) (v) light chain or the fusion sequence from HIV gp41 were joined with or without a WSQP spacer peptide to the NLS from SV40 T-antigen and disulfide conjugated to the 5′-end of a 5′-thiol-substituted, 3′-fluorescein, 26-mer phosphorothioate oligodeoxyribonucleotide through a C-terminal cysteine on the peptide [55]. NIH3T3 fibroblast cells were shown to

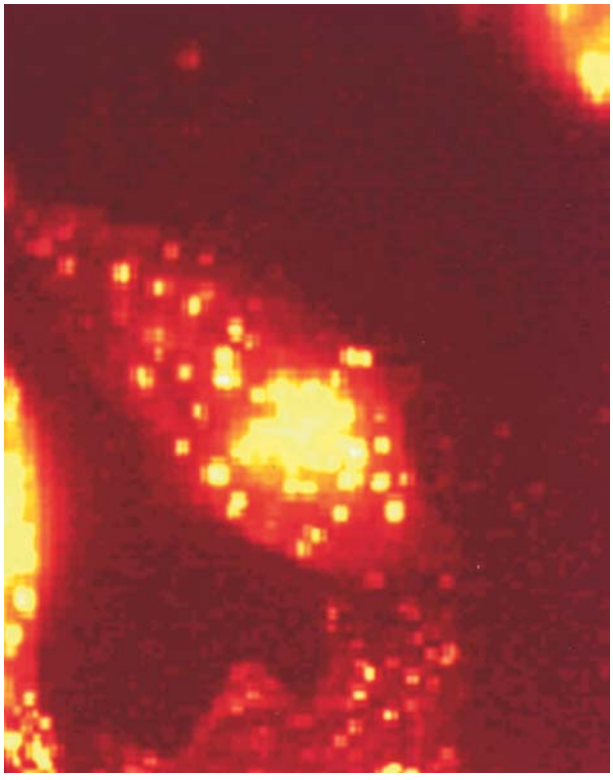

Figure 1. Fluorescence microscopy image and subcellular distribution in MDR-3T3 cells of TAMRA-labelled 20-mer oligodeoxynucleotide phosphorothioate disulfide linked to Tat peptide (see [53] © Elsevier Science).

take up these peptides quite differently depending on the peptide construct. For example, the peptide conjugate containing the *Caiman* Ig-SV40 NLS peptide without spacer had diffuse cellular staining, whilst one with the spacer had a predominantly nuclear location. The former peptide was particularly efficient in directing very rapid (within 5 min) cellular uptake of the conjugate into H9C2 cardiac cells and exhibiting a biological effect. The uptake occurred rapidly both at 37 and 4 °C from which it was deduced that uptake did not involve an endocytotic pathway. Strong nuclear localization was seen under microscopy, but it is not clear whether this visualization was affected by the cell fixation process, in particular the use of concentrated formaldehyde solution followed by 20% acetone washes. Such worries as to uptake mechanism stem from another study of 18 conjugates of peptides and phosphorothioate oligonucleotides linked via either C- or N-terminus through a disulfide linkage to either a hydrophobic signal sequence from K-FGF or to the same domain extended at the C-terminus by a nuclear localization signal (NLS) from transcription factor κB [41]. Fluorescently labelled phosphorothioate oligonucleotide conjugates in all cases showed localization in monkey kidney fibroblast cells CVP-1 predominantly in cytosolic vesicles. The conjugates all failed to show antisense activity but were readily delivered by cationic liposomes into the nucleus.

Thus, apart perhaps from penetratin conjugate delivery into neuronal cells, it seems doubtful that nonendosomal delivery has been achieved for any of the other published peptide-oligonucleotide conjugates involving charged (phosphodiester or phosphorothioate) oligonucleotides. Any antisense activities obtained may therefore be dependent on the abilities of such peptides to release conjugates or detached oligonucleotides from endosomes into other cell compartments in order to block pre-mRNA function or mRNA expression.

Intracellular trafficking of peptide-oligonucleotide conjugates

The inclusion of NLS sequences within a conjugated peptide sequence in order to direct the oligonucleotide to the cell nucleus has already been discussed. In no case has it been suggested that a NLS alone is sufficient to simultaneously enhance the uptake of a negatively charged oligonucleotide into cells from the exterior and deliver into the nucleus. A study of three different types of nuclear signal peptide (the SV40 T-antigen signal, an influenza virus nucleoprotein signal and a signal associated with yeast α2 protein) conjugated through a cysteine residue to the 5′ end of various oligodeoxyribonucleotides proved disappointing [56]. When electroporated into the freshwater ciliate *Paramecium tetraurelia,* all three conjugates showed fivefold less potency in antisense blocking in an assay involving ciliate swimming than an unconjugated oligonucleotide, whereas other 5′-nonsense peptide modifications were well tolerated. No explanation for these results could be proposed. Exciting results were obtained in transfection of a circular DNA duplex that had been conjugated to a single SV40 T-antigen NLS using a novel maleimide modification at the 5′ position of a uracil base [57]. Cationic liposome transfection of a variety of cells (fibroblasts, hepatocytes, HeLa and human macrophages) of an NLS-tagged gene resulted in 100- to 1000-fold enhanced gene expression compared with the untagged DNA. However, no similar studies with antisense oligonucleotides appear to have been published.

More interesting is the finding that a nuclear export signal from the HIV-1 rev protein conjugated via a thioether bond to the 5′ end of a 24-mer phosphodiester oligodeoxyribonucleotide containing a 3′-fluorescein label, when microinjected into the nucleus of HeLa or Vero cells, migrated rapidly to the cytosol [58]. In addition, a similar conjugate of an 18-mer phosphodiester oligonucleotide where the pyrimidines had been modified by hydrophobic C-5 propynes to increase RNA target affinity inhibited expression of a luciferase gene from a T7 pro-

moter that is specifically translated in the cytosol [58]. The conjugate in this case was introduced to the cells by electroporation following plasmid transfection. Thus, routing of oligonucleotides into specific cellular compartments is clearly possible in principle through attachment of certain peptide signals, but this subject is clearly in its infancy.

Cell delivery and activity of PNA-peptide conjugates

By far the most abundant and compelling results have come in the area of delivery and biological activity of peptide conjugates of PNA. PNA is an analogue of nucleic acids where the sugar-phosphate backbone is replaced by spatially similar *N*-(2-aminoethyl)glycine units. This DNA mimic shows enhanced affinity for complementary DNA and RNA sequences without loss of sequence specificity. PNA lacks the negative charge associated with other oligonucleotide analogues and is stable to nuclease degradation. These features make it an attractive analogue for use in antisense studies. Unfortunately, the cellular uptake of unconjugated PNA oligomers is even poorer than normal oligonucleotides, and thus enhancement of uptake is an important goal.

The first reported PNA-peptide conjugate was in 1997 of the D analogue of a short version of insulin-like growth factor 1 (IGF1, Cys-Ser-Lys-Cys) linked through four glycine residues directly to a 12-mer PNA. This conjugate showed much higher uptake into murine cells expressing the IGF1 receptor than unconjugated PNA [59]. In the same year, similar, amide-linked penetratin conjugates with an 11-mer PNA showed dramatically improved uptake into DU145 adherent cells [60]. In this case fluorescent conjugates appeared to be localized in large cytosolic vesicles, and the uptake was independent of peptide orientation. For neither of these peptide-PNA conjugates were activity studies carried out.

Interest in PNA-peptide conjugates received a big stimulus when improved cellular delivery coupled with antisense activity within cells was obtained for a 21-mer PNA coupled to penetratin or transportan through disulfide bonds. These conjugates were directed into human Bowes cells and blocked expression of the galanin receptor [22]. Further, the conjugates were nontoxic when injected intrathecally into rats and showed pain-modifying biological activity. From studies of cells in culture, it was thought that the disulfide bonds were cleaved quickly upon cell entry. Biotin-labelled PNA was uniformly distributed in the cell, whereas differences were observed for cell localization of the peptides. Transportan resided primarily in membrane structures, whereas Penetratin showed a nuclear preference. These results suggested that peptides more stably conjugated to PNA, which are not removed quickly after cell entry, might affect the cellular destination of the PNA. In a more recent example, transportan disulfide-linked to a 16-mer PNA complementary to the HIV-1 TAR RNA sequence blocked Tat-dependent trans-activation in CEM and Jurkat T cells and was found to be efficiently taken up by such cells from the medium, although at varying rates [61]. At least a good part of this PNA uptake can be inferred to be nuclear, since transcriptional trans-activation by Tat is certainly a nuclear event. The PNA-peptide conjugate was also shown to exhibit antiviral activity in chronically HIV-infected H9 cells.

However, stable amide linkages between PNA and peptide have been found also to be compatible with antisense activity in several cases. Such stable linkages are easy to create in the case of PNA peptides merely by continuation of solid-phase synthesis on a single support through use of peptide-type coupling reactions involving both amino acids and PNA monomers [59, 60]. A hydrophobic peptide FLFLFL amide-conjugated to PNA showed increased uptake into mouse macrophages and dose-dependent inhibition of nitric oxide synthase expression [62]. A retroinverso peptide, assembled from D-amino acids and consisting of both hydrophobic and highly basic domains, conjugated to a 16-mer PNA enhanced uptake into neuronal cells and depressed oxytocin mRNA expression [63]. This study is particularly significant because the conjugate was believed to penetrate the neuronal cells by a non-energy-dependent route. A third example is of a NLS peptide PKKKRKV coupled to a 17-mer anti-c-*myc* PNA which showed nuclear localization in BL cells and downregulation of c-*myc* oncogene expression [64]. The cell lines used were derived from Burkitt's lymphoma tissues, and it is particularly interesting that in this case the single NLS sequence was sufficient to allow both uptake into the cell and subsequent predominant passage into the nucleus. However, it should be noted that concentrations of PNA peptide used were relatively high (10 μM) in these experiments. In a complementary study, a PNA-NLS peptide was used as a vehicle to deliver DNA or a synthetic oligonucleotide into the nuclei of Cos-7, HeLa and 3T3 cells [65]. This technique has now been extended to delivery of oligonucleotides to the nuclei of cells in different mouse organs in vivo [66].

Two very recent papers have been particularly informative on the cellular localizatons of certain PNA-peptide conjugates. In the first study, fluorescently labelled Tat peptide or penetratin was conjugated to 14-mer PNA and cellular delivery of each conjugate observed in five cell lines [67]. In both cases, vesicular and diffuse cytoplasmic uptake was seen at 2 μM concentration in media for three cancer cell lines, SK-BR-3, HeLa and IMR-90, whereas rapid and strong cell membrane staining but weak uptake was found for H9 cells, but no uptake into monocytic U-937 cells. No evidence was seen for a nonendosomal, nonendocytotic delivery pathway. Fur-

ther, there was no difference between conjugates linked by an unstable disulfide bond or a stable thiol-maleimide bond. Surprisingly, these authors reported no uptake for either fluorescently labelled Tat or penetratin-free peptides, in contrast to previous studies [12, 68]. The authors also comment that use of strong fixatives in microscopy visualizaton, such as methanol or acetone, leads to misleading results and that only very mild fixative use (short treatment with formaldehyde) or preferably the use of nonfixed cells is essential to allow reliable conclusions to be drawn. No biological activity data were presented in this paper, but the claims of some authors of nonendosomal uptake mechanisms for PNA-peptide conjugates, at least in many standard laboratory cell lines, is now in some doubt, and reevaluation of certain cell uptake localization data may be necessary.

A second recent paper on PNA-peptide cell uptake is also intriguing. A 16-mer PNA was linked to the SV40 T antigen NLS, and a Rhodamine fluorescent label by stable amide bond, which was in turn linked by disulfide bond to a membrane transport peptide (penetratin) and a second (Alexa) fluorescent label. The PNA-NLS section of the conjugate was shown to localize strongly to the nuclei of human prostate cancer cells (DU-145), whilst the penetratin remained in the cytosol, when cells were lightly fixed with formaldehyde and analyzed by confocal laser scanning microscopy (fig. 2A, B) [69]. The exterior concentration of conjugate used was particularly low in these experiments (100 nM). Only cytosolic fluorescence was seen in the absence of the NLS sequence or, interestingly, when the cleavable disulfide bond to the penetratin domain was replaced by a stable amide linkage (fig. 2C). The results suggest that a modular structure of peptide that includes both cell membrane transporter and nuclear transporter is necessary for efficient nuclear delivery from the exterior of the cell. It is not clear yet whether the need for a cleavable bond to the CPP reflects a masking of the NLS sequence that is colinear and adjacent, or some specific cytosolic retention effect of penetratin at these low concentrations used.

Finally, Richard et al. have very recently reevaluated the mechanism of cellular uptake of the HIV-1 Tat peptide by fluorescence microscopy on unfixed cells and shown that the peptide, as well as its conjugates with PNA, showed a characteristic endosomal location and insignificant nuclear penetration [70]. These results challenge previous interpretations of a nonendocytotic mechanism for cell penetration by such cationic peptides and their conjugates.

Inhibition of bacterial cell growth by peptide-PNA conjugates

Good and Nielsen found in 1998 that a triplex-forming PNA targeted to the peptidyl transferase centre of *Escherichia coli* ribosomal RNA could inhibit bacterial translation as well as the growth of an unusually permeable *E. coli* strain [71]. The poor entry of PNA into normal bacterial cells was a disappointment, however. More recent studies have centred on conjugates of PNA with a synthetic cell wall/membrane active peptide $(KFF)_3K$. Dose-dependent reduction of lac-Z reporter gene expression could be obtained in *E. coli* cells after incubation with PNA-peptide constructs [72]. More potent activity was obtained when the PNA and peptide parts were linked through a polyether spacer. Antiribosomal triplex-forming PNA or anti-acpP mRNA duplex-forming PNA, when linked to the carrier peptide, showed minimal inhibitory concentrations of 3 and 0.2 µM, respectively, when incubated with HeLa cells freshly infected with standard *E. coli* K12 cells. In the latter case, the infection was fully cured by treatment with 2 µM PNA-peptide conjugate. These encouraging results may be a prelude to a considerable expansion of effort on PNA-peptide conjugates as antibacterial agents.

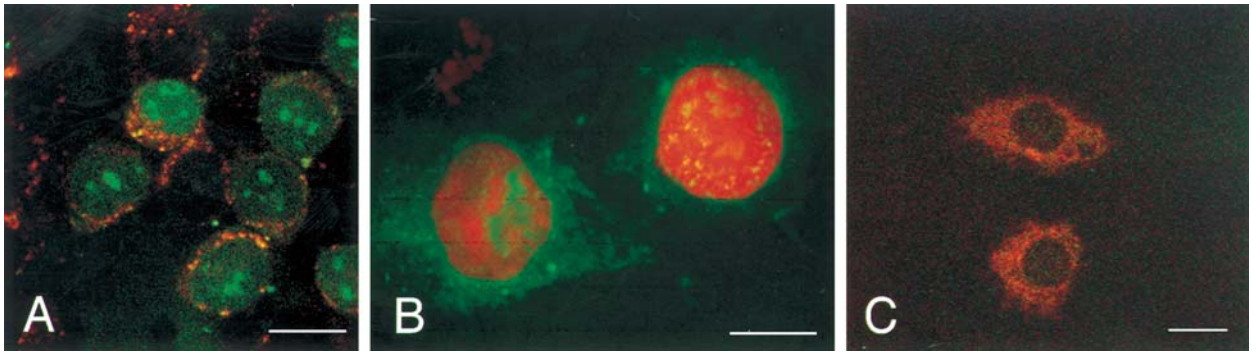

Figure 2. Fluorescence microscopy images of human prostate DU-145 cells incubated with (*A*) Alexa-penetratin-S-S-NLS(Rhodamine)-PNA showing the cytoplasmic distribution of Alexa fluorescence (red) and nuclear fluorescence of Rhodamine (green). Yellow staining represents a mixture of green and red colours. (*B*) reversed chromophores, i.e. Rhodamine-penetratin-S-S-NLS(Alexa)-PNA. (*C*) The NLS(Rhodamine)-PNA is attached to Alexa penetratin by a noncleavable linkage (see [69] © Elsevier Science).

Perspective

The subject of cellular uptake and activity of peptide conjugates of antisense oligonucleotides and their analogues has been full of conflicting and controversial results in recent years. Some of these problems have been due to the complex nature of the experimentation required. As alluded to earlier in this review, there is still insufficient data that addresses both cellular uptake and antisense activity in a range of cell lines, and there are few meaningful conjugate structure-function studies. Further, there are no published results on uptake into primary cell lines in culture and only a few papers that deal with in vivo cell targeting. It seems increasingly clear that the promise of a magic bullet peptide that will deliver all oligonucleotides and analogues into cells by a route that avoids endocytosis and endosomal vesicles, as suggested by some early literature, is probably unattainable, although there is some evidence that certain cargoes may be transportable in this way, especially into neuronal cells. Instead, it is more likely that peptide conjugates can be used more generally as agents to facilitate cell association, cell uptake by endocytotic or other energy-dependent pathways, to enhance release from vesicular endosomes or other cytosolic bodies, and to target to the nucleus, if desired, through use of additional NLS sequences. The use of alternative chemical signals may allow targeting of antisense oligonucleotides or PNA to other organelles such as the mitochondria [73]. It should also be noted that attachment of certain peptides (e.g. cationic) to oligonucleotides or PNA may provide additional advantages not relating to uptake, such as enhanced binding to RNA targets [74]. As methods for the synthesis of conjugates improve, so it is likely that an explosion of literature in this promising field will be forthcoming. Further, peptide conjugation is likely to be extended to synthetic short interfering RNA (siRNA), reagents analogous to antisense which are also now being used for control of gene expression [75], in order to enhance their delivery into cells. It would be surprising if these activities do not lead to a wealth of exciting new discoveries and applications.

Acknowledgements. I thank Bernard Lebleu (CNRS, Montpellier) for valuable comments on the manuscript and several members of my current research group for valuable discussions and comments, namely Andrey Arzumanov, Stephen Holmes, Andrey Malakhov, Birgit Verbeure, Dmitri Stetsenko and Andrew Walsh. I also thank Anna Astriab-Fisher and Rudy Juliano for figure 1 and Klaus Braun for figure 2.

1 Opalinska J. B. and Gewirtz A. M. (2002) Nucleic-acid therapeutics: basic principles and recent applications. Nature Rev. **1:** 503–514
2 Chadwick D. J. and Cardew G. (1997) Oligonucleotides as Therapeutic Agents, John Wiley, Chichester
3 Stein C. A. and Krieg A. M. (1998) Applied Antisense Technology, Wiley-Liss, New York
4 Dean N. M. (2001) Functional genomics and target validation approaches using antisense oligonucleotide technology. Curr. Opin. Biotechnol. **12:** 622–625
5 Bennett C. F., Chiang M.-Y., Chan H., Shoemaker J. E. E. and Mirabelli C. K. (1992) Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. Mol. Pharmacol. **41:** 1023–1033
6 Langel U. (2002) Cell Penetrating Peptides: Processes and Applications, CRC Press, Boca Raton, FL
7 Fischer P. M., Krausz E. and Lane D. P. (2001) Cellular delivery of impermeable effector molecules in the form of conjugates with peptides capable of mediating membrane translocation. Bioconj. Chem. **12:** 825–841
8 Lindsay M. A. (2002) Peptide-mediated cell delivery: application in protein target validation. Curr. Opin. Pharmacol. **2:** 587–594
9 Morris M. C., Chaloin L., Heitz F. and Divita G. (2000) Translocating peptides and proteins and their use for gene delivery. Curr. Opin. Biotechnol. **11:** 461–468
10 Derossi D., Joliot A. H., Chassaing G. and Prochiantz A. (1994) The third helix of the *Antennapedia* homeodomain translocates through biological membranes. J. Biol. Chem. **269:** 10444–10450
11 Derossi D., Chassaing G. and Prochiantz A. (1998) Trojan peptides: the penetratin system for intracellular delivery. Trends Cell Biol. **8:** 84–87
12 Vivès E., Brodin P. and Lebleu B. (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J. Biol. Chem. **272:** 16010–16017
13 Fawell S., Seery J., Daikh Chen L.L., Pepinsky B. and Barsoum J. (1994) Tat-mediated delivery of heterologous proteins into cells. Proc. Natl. Acad. Sci. USA **91:** 664–668
14 Wadia J. S. and Dowdy S. F. (2002) Protein transduction technology. Curr. Opin. Biotechnol. **13:** 52–56
15 Torchilin V. P., Rammohan R., Weissig V. and Levchenko T. S. (2001) Tat peptide on the surface of liposomes afford their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. Proc. Natl. Acad. Sci. USA **98:** 8786–8791
16 Eguchi A., Akuta T., Okuyama H., Senda T., Yokoi H., Inokuchi H. et al. (2001) Protein transduction domain of HIV-1 Tat protein promotes delivery of DNA into mamalian cells. J. Biol. Chem. **276:** 26204–26210
17 Schwarze S. R., Ho A., Vocero-Akbani A. and Dowdy S. F. (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse. Science **285:** 1569–1572
18 Polyakov V., Sharma V., Dahlheimer J. L., Pica C. M., Luker G. D. et al. (2000) Novel Tat-peptide chelates for direct transduction of technetium-99m and rhenium into human cells for imaging and radiotherapy. Bioconjugate Chem. **11:** 762–771
19 Lewin M., Carlesso N., Tung C.-H., Tang X.-W., Cory D., Scadden D. T. et al. (2000) Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. Nat. Biotech. **18:** 410–414
20 Lee H. J. and Pardridge W. M. (2001) Pharmacokinetics and delivery of Tat and Tat-protein conjugates to tissues in vivo. Bioconjugate Chem. **12:** 995–999
21 Silhol M., Tyagi M., Giacci M., Lebleu B. and Vivès E. (2002) Different mechanisms for cellular internalization of the HIV-1 Tat-derived cell penetrating peptide and recombinant proteins fused to Tat. Eur. J. Biochem. **269:** 494–501
22 Pooga M., Soomets U., Hällbrink M., Valkna A., Saar K., Rezaei K. et al. (1998) Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. Nat. Biotech. **16:** 857–861
23 Wadhwa M. S., Collard W. T., Adami R. C., Mckenzie D. L. and Rice K. G. (1997) Peptide-mediated gene delivery: influence of peptide structure on gene expression. Bioconj. Chem. **8:** 81–88

24 McGregor C., Perrin C., Monck M., Camillieri P. and Kirby A. J. (2001) Rational approches to the design of cationic gemini surfactants for gene delivery. J. Am. Chem. Soc. **26:** 6215–6220

25 Wyman T. B., Nicol F., Zelphati O., Scario P. V., Plank C. and Szoka F. C. (1997) Design, synthesis and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. Biochemistry **36:** 3008–3017

26 Niidome T., Ohmori N., Ichinose A., Wada A., Mihara H., Hirayama T. et al. (1997) Binding of cationic a-helical peptides to plasmid DNA and their gene transfer abilities into cells. J. Biol. Chem. **272:** 15307–15312

27 Midoux P., Kichler A., Boutin V., Maurizot J.-C. and Monsigny M. (1998) Membrane permeabilization and efficient gene transfer by a peptide containing several histidines. Bioconj. Chem. **9:** 260–267

28 Junghans M., Kreuter J. and Zimmer A. (2000) Antisense delivery using protamine-oligonucleotide particles. Nucleic Acids Res. **28:** e45

29 Pichon C., Freulon I., Midoux P., Mayer R., Monsigny M. and Roche A.-M. (1997) Cytosolic and nuclear delivery of oligonucleotides mediated by an amphiphilic anionic peptide. Antisense Nucleic Acid Drug Dev. **7:** 335–343

30 Dokka S., Toledo-Velasquez D., Shi X., Wang L. and Rojanasukul Y. (1997) Cellular delivery of oligonucleotides by synthetic import peptied carrier. Pharmaceut. Res. **14:** 1759–1764

31 Morris M. C., Vidal P., Chaloin L., Heitz F. and Divita G. (1997) A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. Nucleic Acids Res. **25:** 2730–2736

32 Hariton-Gazal E., Feder R., Graessmann A., Brack-Werner R., Jans D., Gilon C. et al. (2002) Targeting of non-karyophilic cell-permeable peptides into the nuclei of intact cells by covalently attached nuclear localization signals. Biochemistry **41:** 9208–9214

33 Benimetskaya L., Guzzo-Pernell N., Liu S.-T., Lai J. C. H., Miller P. and Stein C. A. (2002) Protamine-fragment peptides fused to an SV40 nuclear localization signal deliver oligonucleotides that produce antisense effects in prostate and bladder carcinoma cells. Bioconj. Chem. **13:** 177–187

34 Mitchell D. J., Kim D. T., Steinman L., Fathman C. G. and Rothbard J. B. (2000) Polyarginine enters cells more efficiently than other polycationic homopolymers. J. Peptide Res. **56:** 318–325

35 Bachmann A. S., Surovoy A., Jung G. and Moelling K. (1998) Integrin receptor-targeted transfer peptides for efficient delivery of antisense oligodeoxynucleotides. J. Mol. Med. **76:** 126–132

36 Singh D., Bisland S. K., Kawamura K. and Gariépy J. (1998) Peptide-based intracellular shuttle able to facilitate gene transfer in mammalian cells. Bioconj. Chem. **10:** 745–754

37 Tung C.-H. and Stein S. (2000) Preparation and application of peptide-oligonucleotide conjugates. Bioconj. Chem. **11:** 605–618

38 Stetsenko D. A., Arzumanov A. A., Korshun V. A. and Gait M. J. (2000) Peptide conjugates of oligonucleotides as enhanced antisense agents: a review. Mol. Biol. **34:** 852–859

39 Zubin E. M., Romanova E. A. and Oretskaya T. S. (2002) Modern methods for the synthesis of peptide-oligonucleotide conjugates. Russ. Chem. Rev. **71:** 239–264

40 Manoharan M. (2002) Oligonucleotide conjugates as potential antisense drugs with improved uptake, biodistribution, targeted delivery, and mechanism of action. Antisense Nucleic Acid Drug Dev. **12:** 103–128

41 Antopolsky M., Azhayeva E., Tengvall U., Auriola S., Jääskeläinen I., Rönkkö S. et al. (1999) Peptide-oligonucleotide phosphorothioate conjugates with membrane translocation and nuclear localization properties. Bioconj. Chem. **10:** 598–606

42 Antopolosky M. and Azhayev A. (2000) Stepwise solid-phase synthesis of peptide-oligonucleotide conjugates employing Fmoc peptide chemistry. Tetrahedron Lett. **41:** 9113–9117

43 Stetsenko D. A., Malakhov A. D. and Gait M. J. (2002) Total stepwise solid-phase synthesis of oligonucleotide (3′-N)-peptide conjugates. Organic Lett. **4:** 3259–3262

44 Lemaitre M., Bayard B. and Lebleu B. (1987) Specific antiviral activity of poly (L-lysine)-conjugated oligodeoxyribonucleotide sequence complementary to vesicular stomatitis virus N protein mRNA initiation site. Proc. Natl. Acad. Sci. USA **84:** 648–652

45 Leonetti J.-P., Degols G. and Lebleu B. (1990) Biological activity of oligonucleotide-poly (L-lysine) conjugates: mechanism of cell uptake. Bioconj. Chem. **1:** 149–153

46 Degols G., Leonetti J.-P., Bekirane M., Devaux C. and Lebleu B. (1992) Poly(L-lysine)-conjugated oligonucleotides promote sequence-specific inhibition of acute HIV-1 infection. Antisense Res. and Dev. **2:** 293–301

47 Bongartz J. P., Aubertin A. M., Milhaud P. G. and Lebleu B. (1994) Improved biological activity of antisense oligonucleotides conjugated to a fusogenic peptide. Nucleic Acids Res. **22:** 4681–4688

48 Arar K., Aubertin A.-M., Roche A.-C., Monsigny M. and Mayer M. (1995) Synthesis and antiviral activity of a peptide-oligonucleotide conjugates prepared by using Nα-(bromoacetyl)peptides. Bioconj. Chem. **6:** 573–577

49 Pichon C., Arar K., Stewart A. J., Dodon M. D., Gazzolo L., Courtoy P. J. et al. (1997) Intracellular routing and inhibitory activity of oligonucleopeptides containing a KDEL motif. Mol. Pharmacol. **51:** 431–438

50 Rajur S. B., Roth C. M., Morgan J. R. and Yarmush M. L. (1997) Covalent protein-oligonucleotide conjugates for efficient delivery of antisense molecules. Bioconj. Chem. **8:** 935– 940

51 Allinquant B., Hantraye P., Mailleux P., Moya K., Bouillot C. and Prochiantz A. (1995) Downregulation of amyloid precursor protein inhibits neurite outgrowth in vitro. J. Cell Biol. **128:** 919–927

52 Troy C. M., Derossi D., Prochiantz A., Greene L. A. and Shelanski M. L. (1996) Downregulation of Cu/Zn superoxide dismutase lead to cell death via the nitric oxide-peroxynitrite pathway. J. Neurosci. **16:** 253–261

53 Astriab-Fisher A., Sergueev D. S., Fisher M., Ramsay Shaw B. and Juliano R. L. (2000) Antisense inhibition of P-glycoprotein expression using peptide-oligonucleotide conjugates. Biochem. Pharmacol. **60:** 83–90

54 Astriab-Fisher A., Sergueev D., Fisher M., Ramsay Shaw B. and Juliano R. L. (2002) Conjugates of antisense oligonucleotides with the Tat and *Antennapedia* cell-penetrating peptides: effect on cellular uptake, binding to target sequences, and biologic actions. Pharmaceut. Res. **19:** 744–754

55 Chaloin L., Vidal P., Lory P., Méry J., Lautredou N., Divita G. et al. (1998) Design of carrier peptide-oligonucleotide conjugates with rapid membrane translocation and nuclear localization properties. Biochem. Biophys. Res. Commun. **243:** 601–608

56 Reed M. W., Fraga D., Schwartz D. E., Scholler J. and Hinrichson R. D. (1995) Synthesis and evaluation of nuclear targeting peptide-antisense oligodeoxynucleotide conjugates. Bioconj. Chem. **6:** 101–108

57 Zanta M. A., Belguise-Valladier P. and Behr J.-P. (1999) Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. Proc. Natl. Acad. Sci. USA **96:** 91–96

58 Meunier L., Mayer R., Monsigny M. and Roche A. C. (1999) The nuclear export signal-dependent localization of oligonucleopeptides enhances the inhibition of the protein expression from a gene transcribed in the cytosol. Nucleic Acids Res. **27:** 2730–2736

59 Basu S. and Wickstrom E. (1997) Synthesis and characterization of a peptide nucleic acid conjugated to a D-peptide analog ofinsulin-like growth factor 1 for increased cellular uptake. Bioconj. Chem. **8:** 481–488

60 Simmons C. G., Pitts A. E., Mayfield L. D., Shay J. W. and Corey D. R. (1997) Synthesis and membrane permiability of PNA-peptide conjugates. Bioorg. Med. Chem. Lett. **7:** 3001–3006
61 Kaushik N., Basu A., Palumbo P., Nyers R. L. and Pandey V. N. (2002) Anti-TAR polyamide nucleotide analog conjugated with a membrane-permeating peptide inhibits Human Immunodeficiency Virus Type I production. J. Virol. **76:** 3881–3891
62 Scarfi S., Giovine M., Gasparini A., Damonte G., Millo E., Pozzolini M. et al. (1999) Modified peptide nucleic acids are internalized in mouse macrophages RAW 264.7 and inhibit inducible nitric oxide synthase. FEBS Lett. **451:** 264–268
63 Aldrian-Herrada G., Desarménien M. G., Orcel H., Boissin-Agasse L., Méry J., Brugidou J. et al. (1998) A peptide nucleic acid (PNA) is more rapidly internalized in cultured neurons when coupled to a retro-inverso delivery peptide. The antisense activity depresses the target mRNA and protein in magnocellular oxytocin neurons. Nucleic Acids Res. **26:** 4910–4916
64 Cutrona G., Carpaneto E. M., Ulivi M., Roncella S., Landt O., Ferrarini M. et al. (2000) Effects in live cells of a c-myc antigene PNA linked to a nuclear localization signal. Nat. Biotech. **18:** 300–303
65 Brandén L. J., Mohamed A. J. and Smith C. I. (1999) A peptide nucleic acids-nuclear localization signal fusion that mediates nuclear transport of DNA. Nat. Biotech. **17:** 784–787
66 Brandén L. J., Christensson B. and Smith C. I. E. (2001) In vivo nuclear delivery of oligonucleotides via hybridizing bifunctional peptides. Gene Ther. **8:** 84–87
67 Koppelhus U., Awasthi S. K., Zachar V., Holst H. U., Ebbeson P. and Nielsen P. E. (2002) Cell-dependent differential cellular uptake of PNA, peptides and PNA-peptide conjugates. Antisense Nucleic Acid Drug Dev. **12:** 51–63
68 Prochiantz A. (1996) Getting hydrophilic compounds into cells: lessons from homeopeptides. Curr. Opin. Neurobiol. **6:** 629–634
69 Braun K., Peschke P., Pipkorn R., Lampel S., Wachsmuth M., Waldeck W. et al. (2002) A biological transporter for the delivery of peptide nucleic acids (PNAs) to the nuclear compartment of living cells. J. Mol. Biol. **318:** 237–243
70 Richard J. P., Melikov K., Vives E., Ramos C., Verbeure B., Gait M. J. et al. (2003) Cell-penetrating peptides. A re-evaluation of the mechanism of cellular uptake. J. Biol. Chem. **278:** 585–590
71 Good L. and Nielsen P. E. (1998) Inhibition of translation and bacterial cell growth by peptide nucleic acid targeted to ribosomal RNA. Proc. Natl. Acad. Sci. USA **95:** 2073–2076
72 Good L., Awasthi S. K., Dryselius R., Larsson O. and Nielsen P. E. (2001) Bactericidal antisense effects of peptide-PNA conjugates. Nat. Biotech. **19:** 360–364
73 Muratovska A., Ligtowlers R. N., Taylor R. W., Turnbull D. M., Smith R. A. J., Wilce J. A. et al. (2001) Targeting peptide nucleic acid (PNA) oligomers to mitochondria within cells by conjugation to lipohilic cations: implications for mitochondrial DNA replication, expression and disease. Nucleic Acids Res. **29:** 1852–1863
74 Corey C. R. (1995) 48000-fold acceleration of hybridisation by chemically modified oligonucleotides. J. Am. Chem. Soc. **117:** 9373–9374
75 Elbashir S. M., Martinez J., Patkaniowska A., Lendeckel W. and Tuschl T. (2001) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J. **20:** 6877–6888