Improved cell-penetrating peptide–PNA conjugates for splicing redirection in HeLa cells and exon skipping in mdx mouse muscle

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ABSTRACT
Steric blocking peptide nucleic acid (PNA) oligonucleotides have been used increasingly for redirecting RNA splicing particularly in therapeutic applications such as Duchenne muscular dystrophy (DMD). Covalent attachment of a cell-penetrating peptide helps to improve cell delivery of PNA. We have used a HeLa pLuc705 cell splicing redirection assay to develop a series of PNA internalization peptides (Pip) conjugated to an 18-mer PNA705 model oligonucleotide with higher activity compared to a PNA705 conjugate with a leading cell-penetrating peptide being developed for therapeutic use, (R-Ahx-R)₄. We show that Pip–PNA705 conjugates are internalized in HeLa cells by an energy-dependent mechanism and that the predominant pathway of cell uptake of biologically active conjugate seems to be via clathrin-dependent endocytosis. In a mouse model of DMD, serum-stabilized Pip2a or Pip2b peptides conjugated to a 20-mer PNA (PNADMD) targeting the exon 23 mutation in the dystrophin gene showed strong exon-skipping activity in differentiated mdx mouse myotubes in culture in the absence of an added transfection agent at concentrations where naked PNADMD was inactive. Injection of Pip2a-PNADMD or Pip2b-PNADMD into the tibialis anterior muscles of mdx mice resulted in ~3-fold higher numbers of dystrophin-positive fibres compared to naked PNADMD or (R-Ahx-R)₄-PNADMD.

INTRODUCTION
Sequence-specific steric blocking oligonucleotides (ON) that target intra-cellular RNAs have excellent potential for development as therapeutic agents for a variety of diseases (1,2). In contrast to standard antisense or siRNA, there is no requirement for recognition of the ON–RNA hybrid by a cellular enzyme complex (such as RNase H or RISC) in order to achieve biological activity. Instead, the ON is targeted at a specific RNA site to inhibit or alter an essential function or protein recognition merely by ON–RNA hybrid formation and resultant steric interference. This approach may have higher specificity than those dependent on RNA cleavage since binding at an incorrect site is less likely to trigger a biological effect. Further it allows a greater variety of ON chemistry to be explored and hence a better opportunity to adjust both cell delivery and pharmacology.

The steric block approach is particularly useful to interfere with specific pre-mRNA processing in the cell nucleus and hence to alter gene expression. For example, a number of clinically relevant applications involve the redirection of splicing, where ONs are targeted at a splice site or at splicing regulating sequences (3). The most clinically advanced disease target of this type is Duchenne muscular dystrophy (DMD). DMD is an X-linked muscle disorder caused mainly by nonsense or frame-shift mutations in the dystrophin gene, occurring with a frequency of about one in 3500 live male births. DMD patients suffer from severe, progressive muscle wasting, whereas the milder Becker muscular dystrophy (BMD) is caused by in-frame deletions resulting in expression of a shortened but partially functional protein. ONs have been shown to induce targeted ‘exon skipping’ to correct the reading frame of mutated dystrophin mRNA such that shorter dystrophin forms are produced with activity similar to that of BMD (4).

Many types of ON have been investigated in a mouse muscle cell model and also in an mdx dystrophic mouse model, where a nonsense mutation in exon 23 is skipped to restore dystrophin production (5–8). Initially 2'-O-methyl phosphorothioate (2'OMePS) ONs were used to target the human dystrophin gene (9,10). This backbone has been
taken to a Phase I clinical trial in Holland targeting exon 51 of dystrophin pre-mRNA in DMD patients involving intramuscular injection with promising results in localized dystrophin production (11). A similar Phase I trial is currently in progress in the UK involving use of a 30-mer phosphorodiamidate morpholino oligonucleotide (PMO) (12). Studies in vivo have suggested higher levels of exon skipping and restoration of dystrophin expression using PMO compared to 2′OMePS (8,12). PMOs are non-ionic ONs and are less likely to form unwanted interactions with other intra-cellular molecules of target cells. PMOs have been used in animal models of disease and several clinical trials to date (13). Very recently Yin et al. (14) have demonstrated that use of a second non-ionic ON type, known as peptide nucleic acid (PNA), also leads to a significant increase in the number of dystrophin-positive fibres when PNA targeted to the exon 23 mutation was injected into the tibialis anterior (TA) muscles of mdx mice, and with a higher efficiency than a naked 2′OMePS ON.

However, a key issue in use of ONs as therapeutics has been to achieve a sufficient level of intra-cellular delivery, especially in vivo for example within diseased muscle of DMD patients, such that the ON is in significant excess over the RNA target and remains so in order to achieve a high and sustained level of biological activity. Conjugation of the ON to a cell-penetrating peptide (CPP) enhances significantly the activity of both PNA and PMO in cellular and animal models (15–19). In the case of PMO, an arginine-rich lead peptide has been proposed, (R-Ahx-R)x-Ahx-β-Ala (or RXR4XB), where Ahx (X) is aminohexanoyl. This peptide takes into account the key roles played by Arg side chains in CPP uptake. Several examples of enhanced activity of RXR4XB-PMO over naked PMO have been published in both cell and animal models (2,13), including recently in DMD studies through intraperitoneal injection into mdx mice (20).

To assess the intra-nuclear activity levels of CPP-ON conjugates, we have used a well-established HeLa cell assay that involves splicing redirection of an aberrant β-globin intron by an 18-mer synthetic ON (targeted to the 705 site) and subsequent upregulation of firefly luciferase (21). This assay is straightforward and has a high dynamic range, allowing both high- and low-activity levels to be measured quantitatively as a positive luminescence read-out. In addition the EC50 of the splicing redirection can be assessed readily at the RNA level by an RT-PCR assay. We showed that an RXR4XB-PMO705 conjugate had significant splicing redirection activity in the luciferase upregulation model at 1 μM concentration (22). Similarly we showed that a RXR4-PNA705 conjugate also had significantly better splicing redirection activity (23) than could be achieved with other well-known CPPs, such as Tat, Penetratin, R9 or K8 (24–26). We (23,26–29) and other groups (30,31) have shown that the major barrier for nuclear delivery of CPP–PNA, required for splicing redirection, is release from endocytotic vesicular compartments. Indeed, polycationic CPPs are internalized by an active mechanism of endocytosis, which involves electrostatic interactions with cellular heparan sulphates, and have little access to the nuclear compartment (27). Thus, for many standard CPPs conjugated to PNA, endosomolytic agents, such as chloroquine, calcium ions or high sucrose concentration (28,32), are necessary to obtain a significant splicing redirection activity (23–26). Therefore, the key to improving activity levels further is to search for CPPs that can trigger enhanced endosomal release.

We described recently R6-Penetratin (R6Pen), a derivative of Penetratin in which six Arg residues were added to the N-terminus of the CPP. Activity was observed in an HIV-1 trans-activation inhibition assay that requires nuclear delivery when R6Pen was disulphide-conjugated to a PNA complementary to the trans-activation responsive element RNA (28). We also showed that R6Pen disulphide or stably conjugated to PNA705 gave significantly better upregulation of luciferase in the splicing redirection assay than a number of other CPP–PNA705 conjugates, including RXR4-PNA705, at both protein and RNA levels and showed an EC50 of ~1 μM (33).

Starting with the R6Pen lead, we have now designed a series of PNA internalization peptides (Pip) with retained or improved activity in the HeLa cell splicing redirection assay when conjugated to PNA705 and which are better stabilized to serum proteolysis. We show that Pip–PNA705 conjugates are internalized in HeLa cells by an energy-dependent mechanism and predominantly via clathrin-dependent endocytosis. Conjugates of Pip2a or Pip2b to a 20-mer PNA targeted to the exon 23 mdx mutation (PNADMD) showed strong exon-skipping activity in differentiated mdx mouse myotubes and a higher number of dystrophin-positive fibres when injected into the TA muscles of mdx mice compared to naked PNADMD or RXR4-PNADMD.

**MATERIALS AND METHODS**

**Synthesis of peptide–PNA conjugates**

**Synthesis of PNA.** N-terminal nitropyridyl (Npys) cysteine-containing PNA705 (NH2-Cys(Npys)-Lys-CCT CTACCTCAGTTACA-Lys-amide) was synthesized on an Apex 396 Synthesizer (Advanced ChemTech) or on a Liberty microwave peptide synthesizer (CEM) by the Fmoc/Bhoc method as previously described (28,34). Batches of naked 20-mer PNADMD (GGCCAAAC CTGGGCTACCT) and RXR4-PNADMD, were obtained from Panagene (Korea) or synthesized in house on a Liberty microwave peptide synthesizer. N-terminal bromoacetylation was carried out by the method previously reported (35).

MALDI-TOF mass spectrometry was carried out on a Voyager DE Pro BioSpectrometry workstation with a matrix of α-cyano-4-hydroxycinnamic acid, 10 mg ml−1 in acetonitrile/3% aqueous trifluoroacetic acid (1:1, v/v). The accuracy of the mass measurement in linear mode is regarded by the manufacturer as ±0.05%, but since internal calibration was not used, the determined values varied in a few cases from the calculated by ±0.1%.
RT-PCR analysis of splicing redirection

For dose-dependence experiment, cells were treated as described above with increasing concentrations of conjugates. After carrying out the luciferase assay and BCA™ Protein Assay, the remaining cell lysates (about 270 μl) were transferred into 2-ml microfuge tubes and total RNA was extracted with 1 ml TRI Reagent (Sigma). Minor changes to the manufacturer’s protocol were made to accommodate the presence of Reporter Lysis Buffer. Thus, 0.3 ml of chloroform was used for extraction and the amount of isopropanol for RNA precipitation was increased to give a 1:1 mixture with the aqueous phase. The extracted RNA was examined by RT-PCR (Genius Techne Thermal cycler) with forward primer 5'-TTG ATA TGT GGA TTT CGA GTC GTC-3' and reverse primer 5'-TGT CAA TCA GAG TGC TTT TGG CG-3'. The products were analysed on a 2% agarose gel, which was scanned using Gene Tools Analysis Software (SynGene, Cambridge, UK).

Mechanism assays

For studies of energy-dependence, HeLa pLuc705 cells were grown as usual and pre-incubated for 30 min in OptiMEM at 37°C or 4°C. CPP–PNA conjugates were then added at a final concentration of 1 μM and incubation was continued for 1 h. Luciferase expression was monitored as described in the splicing redirection assay section.

For studies of the endocytotic pathway, HeLa pLuc705 cells were grown as usual and pre-incubated for 30 min in OptiMEM at 37°C in the presence of the appropriate inhibitors. CPP–PNA conjugates were then added at a final concentration of 1 μM and incubation was continued for 30 min. Luciferase expression was monitored as described in the splicing redirection assay section. Chlorpromazine (30 μM) or K⁺ depletion were used to inhibit clathrin-coated pits-mediated endocytosis; nystatin (50 μM) or filipin-III (5 μg ml⁻¹) were used to inhibit caveola-mediated endocytosis; methyl-β-cyclodextrin (mBCD) (2.5 mM) or 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) (1 mM) were used to inhibit macropinocytosis. Endocytosis inhibitors were used at concentrations that did not affect significantly cell metabolism, as judged by the absence of effect on protein synthesis levels (data not shown).

Serum stability assay

CPP–PNA conjugates (20 μM) were incubated in PBS containing 20% mouse serum (prepared by 3 × 30 min 13 000 r.p.m. centrifugation at 4°C of fresh clotted blood from female Balb/c mice) at 37°C. Aliquots of 10 μl were taken at 0, 15, 30, 60 and 120 min and diluted with 50 μl 10% dichloroacetic acid (DCA) in H₂O/CH₃CN (50/50). The samples were mixed and kept at –20°C. The precipitated serum proteins were separated by centrifugation (13 000 r.p.m., 5 min) and the supernatant was analysed by MALDI-TOF mass spectrometry.
Exon skipping in mouse mdx muscle cells

H2K mdx myoblasts were cultured at 33°C under a 10% CO₂ atmosphere in DMEM supplemented with 20% FBS, 1.5% chicken embryo extract (PAA Laboratories Ltd, Yeovil, UK), and 20 U ml⁻¹ Interferon γ (Invitrogen). Myotubes were obtained from confluent H2K mdx cells seeded in gelatin-coated 12-well plates after 3 days of serum deprivation at 37°C under a 5% CO₂ atmosphere (DMEM with 5% horse serum). The CPP–PNA conjugates were incubated with myotubes for 4 h in 1 ml OptiMEM and then replaced by 2 ml of DMEM/5% horse-serum media for further incubation. After 20 h myotubes were washed twice with PBS and total RNA was extracted with 1 ml of TRI Reagent. RNA preparations were treated with RNase free DNase (2 U) and Proteinase K (20 μg) prior to RT-PCR analysis. The RT-PCR was carried out in 25 μl with 1 μg RNA template using SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen) primed by forward primer 5'-CAG ATT CTC GCC AAT TGC TGAG-3' and reverse primer 5'-TTC TTC TAC AGC TTG TGT CAT CC-3'. The initial cDNA synthesis was performed at 55°C for 30 min followed by 30 cycles of 95°C for 30 s, 55°C for 1 min and 68°C for 80 s. RT PCR product (1 μl) was then used as the template for secondary PCR performed in 25 μl with 0.5 U Super Taq polymerase (HT Biotechnologies) and primed by forward primer 5'-CCC AGT CTA CCA CCC TAT CAG AGC-3' and reverse primer 5'-CGT GCC TTT AAG GCT TCC TT-3'. The cycling conditions were 95°C for 1 min, 57°C for 1 min and 72°C for 80 s for 25 cycles. Products were examined by 2% agarose gel electrophoresis.

For the electroporation assays, non-differentiated myoblast cells were seeded 2 days before the electroporation and maintained in 20% FBS/DMEM with Interferon γ at 33°C in 10% CO₂. Cells were treated with trypsin, counted and centrifuged at 1000 r.p.m. for 5 min, then resuspended in 0.5 ml of Nucleofactor Solution V (Amaxa, Gaithersburg, MD, USA) to give 1.5 × 10⁶ cells/100 μl. 100 μl of cell suspension was mixed with 0.5 nmol of PNA or PNA–peptide conjugate and placed into an Amaxa cuvette. After an electric pulse (program T-020) the cell suspension was mixed with 0.5 ml OptiMEM and incubated in a microfuge tube for 10 min at room temperature. Cells were then transferred into a gelatin-precoated 6-well plate containing 4 ml of 20% FBS/DMEM/Interferon γ and incubated for 24 h at 33°C in 10% CO₂. After two washes with PBS total RNA was extracted with 1.5 ml of TRI Reagent and the PCR carried out as above.

For the cell viability assay (data not shown), myotubes in gelatin-coated 24-well plates were incubated with CPP–PNA conjugates for 4 h in 0.5 ml OptiMEM followed by a further 20-h incubation in 1 ml of DMEM/5% horse serum. Colorimetric MTS cell viability assay was performed using 200 μl/well of CellTiter 96 Aqueous One solution Cell Proliferation Assay (Promega). Each data point was averaged over duplicates of three experiments.

Animals and intramuscular injection

Six- to eight-week-old mdx mice were used in all experiments (three mice each in the test and five in control groups). The tibialis anterior (TA) muscle of each experimental mdx mouse was injected with 5 μg of PNA or PNA–peptide conjugate in 40 μl of saline at a final concentration of 125 μg ml⁻¹, and the contralateral muscle was injected with saline. The experiments were carried out in the Animal Unit, Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK, according to procedures authorized by the UK Home Office. Mice were killed by cervical dislocation 2 weeks after injection, and muscles were snap-frozen in liquid nitrogen-cooled isopentane and stored at −80°C.

Immunohistochemistry. Sections of 8 μm were cut from at least two-thirds of the muscle length of TA at 100 μm intervals. The sections were then examined for dystrophin expression with a polyclonal antibody 2166 against the dystrophin carboxyl-terminal region (the antibody was kindly provided by Professor Kay Davies). The intervening muscle sections were collected either for RT-PCR analysis and western blot or as serial sections for immunohistochemistry. Polyclonal antibodies were detected by goat-anti-rabbit IgGs. Alexa Fluro 594 (Molecular Probe, UK) and nuclei were counter-stained with DAPI. Dystrophin-positive fibres were counted by fluorescence microscopy using Alexa vision LE software and presented per nanomole compound.

RNA extraction and nested RT-PCR analysis. Total RNA was extracted from TA muscle tissue with Trizol (Invitrogen, UK) and 800 ng of RNA template was used for 20 μl RT-PCR with the OneStep RT-PCR kit (Qiagen, UK). The primer sequences for the initial RT-PCR were as shown above for muscle cell studies and were used for amplification of messenger RNA from exons 20 to 26. The cycle conditions were 95°C for 30 s, 55°C for 1 min and 72°C for 2 min for 25 cycles. RT-PCR product (1 μl) was then used as the template for secondary PCR performed in 25 μl with 0.5 U Taq DNA polymerase (Invitrogen, UK). The primer sequences for the second round were the same as shown above for muscle cell studies. The cycle conditions were 95°C for 1 min, 57°C for 1 min and 72°C for 2 min for 25 cycles. The products were examined by electrophoresis on a 2% agarose gel.

RESULTS

We chose to use the HeLa cell splicing redirection assay (33) to monitor the activity levels of derivatives of R6Pen–PNA705 altered in the peptide part. It is convenient to consider the 25-mer R₆Penetratin (R6Pen) peptide as comprising three segments (Figure 1a), an N-terminal oligo–Arg region (segment 1), a central more hydrophobic region (segment 2) and a C-terminal more basic region (segment 3). R6Pen is linked to the PNA via a short Gly–Gly spacer that is conjugated to the PNA via a reducible disulphide or stable thioether linkage to the terminal Cys residue. The resultant conjugate has an EC₅₀ of about
1 μM in splicing redirection by RT-PCR analysis after incubation with HeLa pLuc705 cells (33). In preliminary studies we found that replacement of Trp by Leu in segment 2 resulted in a slight improvement in splicing redirection (33). When this W→L mutation was combined with replacement of segment 1 by (R-Ahx-R)₃ (i.e. spacing of the six Arg residues by three aminohexanoyl linkers, shown as X in Figure 1a), the activity increased further (37). Since three non-natural amino acids had been added, we shortened the peptide by deletion of RQ from segment 1 and removed the GG spacer, to give the 24-mer PNA Internalization Peptide 1 (Pip1, Figure 1a). Pip1 disulphide-conjugated to 18-mer CKPNA705 (PNA705) was tested in the HeLa cell splice redirection assay for luciferase activity (Figure 1b) and by RT-PCR analysis (Figure 1c) for amounts of aberrant and correctly spliced RNA. Pip1-PNA705 showed two to three times the fold increase in luminescence compared to R6Pen–PNA705 at similar concentrations (Figure 1b). The EC₅₀ in the RT-PCR assay for Pip1–PNA705 was 0.50±0.05 μM, which was about 2-fold better than R6Pen–PNA (Figure 1c) (33). RXR4–PNA705 was substantially less active in both assays with an EC₅₀ of splice redirection of 3–4 μM (Figure 1c). We also synthesized a conjugate of PNA705 with (R-Ahx-R)₄-Ahx-Cys (RXR4X), which contains an additional Ahx (X) spacer, and the EC₅₀ of this conjugate in splice redirection was very similar to that of RXR4-PNA705 (data not shown).

**Serum stabilized Pip peptides as PNA705 conjugates**

For in vivo studies, it is necessary to ensure that the peptide component of the conjugate is sufficiently stable to serum proteolysis so that there is a better chance of the conjugate reaching the necessary cells. We therefore developed a convenient assay based on incubation of conjugates with 20% mouse serum, observation of the loss of conjugate and determination of the fragment masses of proteolytic cleavage products by MALDI-TOF mass spectrometry. Although not fully quantitative, it is possible to identify the most vulnerable proteolysis sites very easily.
from the masses of the cleavage fragments. For example, Pip1–PNA was fully cleaved within 1 h under these conditions, with the major fragment observed being consistent with cleavage between amino acids R_{17} and R_{18} (Figure 2a). Minor cleavages were observed between K_{11} and I_{12}, between K_{22} and K_{23} and between K_{23} and C_{24} (data not shown). It should be noted that no cleavage was observed within the PNA and insignificant cleavage at the disulphide linkage occurred under these conditions. Under the same serum incubation conditions, R6Pen–PNA705 was heavily degraded in the peptide component within a few minutes and cleavage was also observed between the same amino acid residues (data not shown).

A series of peptides was then synthesized iteratively aimed at altering the sequences at the identified vulnerable regions to increase their stability to serum proteolysis, but without losing significant splice redirection ability when disulphide-conjugated to PNA705. For each peptide in the series, the EC_{50} of the conjugate was measured in the RT-PCR assay and the serum stability studied by MALDI-TOF mass spectrometry (Supplementary Table 1). The culmination of this study was two candidate peptides, Pip2a ((R-Ahx-R)_3IdKILFQNdRRMKWHKB) and Pip2b ((R-Ahx-R)_3IHILFQNdRRMKWHKBC), differing only in a single amino acid in position 11 (Supplementary Table 1). Conjugates of these peptides with PNA705 were predominantly stable for 1 h in 20% mouse serum (Figure 2b and Supplementary Table 1). RXR4–PNA705 was found also to be predominantly stable under the same serum conditions as shown in Figure 2c. In the HeLa pLuc705 cell splicing redirection assay the EC_{50} for Pip2a–PNA705 was 0.79 ± 0.13 μM and for Pip2b–PNA705 was 0.64 ± 0.07 μM (Supplementary Table 1), intermediate between that of Pip1–PNA705 and R6Pen–PNA705.

Cell uptake mechanism of Pip–PNA705 conjugates

The increased splicing redirecting activity of Pip–PNA conjugates as compared to R6Pen–PNA705 and RXR4–PNA705 could be due to differences in their cellular uptake mechanism. Several publications have indeed pointed to non-endocytotic mechanisms or macroinocytosis as more favourable pathways. We first verified that all CPP–PNA conjugates in this series were taken up by an energy-dependent mechanism (Figure 3a). In order to delineate which endocytotic pathway was prevalent, two well-characterized inhibitors of each route were used. Chlorpromazine (30 μM) or K⁺ depletion were used to inhibit clathrin-coated pits-mediated endocytosis. Nystatin (50 μM) or fillipin-III (5 μg ml⁻¹) were used to inhibit caveolae-mediated endocytosis. Methyl-β-cyclo-dextrin (2.5 mM) or 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) (1mM) was used to inhibit macroinocytosis. Since these inhibitors are not devoid of cytotoxicity, the concentrations and the duration of pre-incubation and of incubation were optimized to avoid any significant effect on protein synthesis (data not shown). As shown in Figure 3b for Pip2b–PNA705, chlorpromazine and K⁺ depletion decreased luciferase expression by 90% and 74%, respectively, in keeping with clathrin-coated
dextrin, EIPA = 5-([N-inhibitor experiment. Cpz = chlorpromazine, mBCD = methyl-

are shown as relative light units per microgram protein, rather than

The results

an energy-dependent uptake mechanism. (b) The effect of endocytosis

pits being the major pathway of internalization. Caveolae

inhibitors such as nystatin or fillipin-III had lower

Macropinosis did not seem to be involved since methyl-

and for RXR4–PNA or RXR4–PMO conjugates [(22) and

conjugates thus appear to be internalized mainly through

Synthesis of Pip–PNA conjugates targeted to dystrophin

pre-mRNA and exon-skipping activity in cultured mdx

muscle cells

Pip1, Pip2a, Pip2b and RXR4 peptides were each conjugated via a stable thioether linkage to a 20-mer PNA (PNADMD) that was shown previously to have activity as naked PNA in exon 23 skipping in the mdx mouse by direct muscle injection (14). These conjugates were incubated for 4 h with differentiated mdx mouse myotubes at 1 or 2 μM concentrations without any other transfection system. RNA was isolated after 20 h from the cells and nested RT-PCR analysis was carried out to measure the levels of exon skipping (Figure 4). Pip1–PNADMD and RXR4–PNADMD each showed a small amount of exon skipping at 2 μM, but Pip2a–PNADMD and Pip2b–PNADMD showed significantly higher levels of exon skipping at both 1 and 2 μM concentrations. No exon skipping was observed for naked PNADMD. This shows that in the absence of any transfection method Pip2a and Pip2b peptide conjugation enhances substantially the cell and nuclear delivery of PNADMD into mdx muscle cells as compared to the other peptides. Specificity of exon skipping by PNADMD compared to a sense control PNA was observed by electroporation of 0.5 nmol quantities into the mdx myoblasts. Naked PNADMD, Pip2a–PNADMD and Pip2b–PNADMD each showed exon skipping as expected when electroporated into the cells, whereas the sense control PNA showed no exon skipping (Supplementary Figure 1).

Cell viability was checked by a standard MTS assay for Pip2a–PNADMD and Pip2b–PNADMD. No significant loss of viability was seen for mdx mouse muscle cells treated with 2 μM conjugate and the viability at 5 μM was ~80–85% (data not shown).

**Exon skipping and dystrophin expression following intramuscular injection in the mdx mouse**

The exon-skipping effects of naked PNADMD and PNADMD–peptide conjugates were evaluated in mdx mice by local intramuscular injection. Six- to eight-week-old mdx mice (referred to as 8 weeks) were injected with a single dose of 5 μg PNA or PNA–peptide conjugate into the TA muscle. Two weeks after injection all treated TA muscles were harvested and dystrophin-positive fibres were identified by immunohistochemistry (Figure 5a). Inspection of whole-muscle transverse sections after a single injection of PNA or PNA–peptide conjugates showed widespread and uniform distribution of dystrophin-positive fibres throughout the muscle cross-sections, except in the cases of Pip1–PNADMD-treated mice, in which only a limited number of dystrophin-positive fibres was observed, and the untreated mdx mouse control (Figure 5a). Of particular interest were Pip2a–PNADMD and Pip2b–PNADMD-treated mdx muscles which showed a highly significant increase ($P < 0.05$) in the number of dystrophin-positive myofibres compared with those treated with PNA alone, Pip1 and RXR4–PNA conjugates compared with age-matched control mdx mice (Figure 5b). Consistent with these immunohistochemistry data, RT-PCR analysis showed the expected exon 23 skipped bands (Figure 5c).

**DISCUSSION**

Pip2a and Pip2b are representatives of a new class of cell-penetrating peptide with enhanced serum-stability and higher cell delivery potential. We chose PNA (over PMO) as the ON cargo type because of its ready synthetic accessibility and ability to be conjugated to peptides by either disulphide or stable thioether linkages. Pip2a- and Pip2b–PNA conjugates showed higher activity than
conjugates of the well-known RXR4 peptide in redirection of splicing in HeLa cells and in exon skipping in mouse mdx muscle cells. The activity improvement was paralleled in vivo in a mouse mdx TA muscle injection model. Pip2a and Pip2b are composed of 3 peptide segments containing an Arg-rich domain, a hydrophobic segment and a broadly cationic segment. However, the structure-activity data to date from the HeLa pLuc705 experiments in this work and previous studies (33,37) point to a more subtle relationship between peptide sequence and splicing.

Figure 4. Exon skipping in mouse mdx muscle myotubes for naked PNADMD, RXR4-PNADMD, Pip1-PNADMD, Pip2a-PNADMD and Pip2b-PNADMD at 1 and 2 μM in the absence of a transfection agent.

Figure 5. In vivo activity of Pip–PNA conjugates following intramuscular injection in mdx mice. Mouse i.m. injections for PNADMD and its peptide conjugates: (a) immunostaining of TA muscle cross-sections to detect dystrophin positive myofibres; (b) quantification of the number of dystrophin-positive fibres; and (c) RT-PCR analysis of exon skipping following intramuscular injection. C57 = control non-dystrophic mouse.
redirection than a mere juxtaposition of two cationic segments surrounding a hydrophobic segment. Within each segment there appear to be further sequence selectivities. For example, aminohexanoyl spacing within segment 1 is advantageous and some spacing of cationic residues in segment 3 also seems important (37). Note that activity levels are not related to the presence of a pH sensitive His residue in segment 3, since Lys in this position gives equal activity (Supplementary Table 1). We also reported previously that the efficiency of splicing redirection for R6Pen-PNA705 does not appear to be correlated with the membrane crossing potential of the Penetratin peptide itself (33).

Serum-stabilized Pip2a and Pip2b provided a good starting point for in vivo studies. These 25-mer peptides were several times more active as conjugates of PNA705 compared to the 15-mer RXR4 peptide, which is the leading peptide used for in vivo studies with PMO conjugation (13,20), whilst showing similar stability to serum proteolysis under the conditions studied (Figure 2). The significantly higher activity levels observed in both muscle cell and in vivo assays for the longer Pip2 peptide (25-mer) compared to the shorter RXR4 (15-mer) justifies the use of a longer peptide. However, additional structure–function analysis may yet identify shorter variants with maintained or enhanced activity.

We showed recently that splicing redirection activity of a PMO705 conjugate of RXR4 (39) and a PNA705 conjugate of R6Pen (37) had much lower activities when incubated with HeLa pLuc705 cells at 4°C compared to 37°C (Figure 3a), suggesting in each case an energy-dependent cell uptake mechanism. We showed here in side-by-side comparison that both Pip2b and RXR4, when conjugated to PNA705 also appear to have energy-dependent uptake pathways, suggesting that this is a general property imparted by Arg-rich CPPs.

We and others have shown that ONs conjugated to cationic CPPs bind to heparan sulphate on the surface of HeLa cells and upon entry appear to become trapped within endocytotic vesicles (26,28,30,38). Our current study is of the effect of endocytosis inhibitors on splicing redirection (Figure 3b) and is therefore focused on the biologically active part of the conjugate that is undergoing cellular uptake. These results show clearly, and for the first time, that in HeLa pLuc705 cells clathrin-dependent endocytosis seems to be the major uptake route for biologically available Pip2b–PNA705 conjugate. The implementation of an energy-dependent endocytic pathway of cellular uptake, as opposed to direct membrane translocation, for most CPPs is still the object of debate. Most studies have relied on fluorescently labelled ONs and observation of the cellular locations by microscopy and the effect of endocytosis inhibitors on such uptake and localization (40,41), rather than a biological assay, as carried out here. Likewise, controversies still exist concerning which endocytotic pathway, macropinocytosis (40,41) or clathrin-coated pits (38 and this study), is the major route of internalization for cationic CPPs. The cases for each of these pathways have been laid out in a recent review of cellular uptake of CPPs (42). Again we feel that monitoring a biological response in the presence of a range of inhibitors is more relevant than relying on the uptake of fluorescent conjugates.

In preliminary studies, co-incubation of HeLa pLuc705 cells with 200 nM CPP-PNA705 conjugates and a cell-permeabilizing agent saponin led to substantial increases in luciferase production resultant from splicing redirection for both Pip2b–PNA705 and RXR4–PNA705, with the former being only marginally more active than the latter (data not shown). By contrast there is at least a 3- to 4-fold higher activity of Pip2b–PNA705 compared to RXR4–PNA705 in the absence of a transfection or permeabilization agent. This suggests that endosomal trapping is a main limitation for splicing redirection activity and that the Pip2b peptide may therefore trigger a better endosomal release than RXR4. Further experiments to confirm these observations with a range of CPP–PNAs under a variety of cell conditions are in progress and results will be reported later.

In the absence of any transfection agent, Pip2a–PNADMD and Pip2b–PNADMD showed significant exon-skipping activity in mouse mdx myotubes in the low micromolar range whereas RXR4–PNADMD showed only slight activity (Figure 4). Despite the quantitative differences between splicing redirection and exon skipping levels, the correlation between enhancements of activity for PNA conjugates of Pip peptides over RXR4 peptide in the HeLa cell and mouse muscle cell models is reassuring and demonstrates that better cell permeation and nuclear delivery is a general property imparted by the Pip peptide series. A similar correlation seems also to hold in vivo, based on immunohistological analysis of mouse TA muscles treated with Pip2a–PNADMD or Pip2b–PNADMD compared to RXR4–PNADMD (Figure 5a and b). The relatively poor activity of Pip–PNADMD in mdx muscle cells and in vivo, in contrast to the HeLa cell result for Pip1–PNA705, presumably reflects the need for better proteolytic stability of the CPP in the mdx muscle system, since cell and in vivo data appear to correlate well.

The low levels of exon skipping at the RNA level observed for all constructs after a single local injection into the TA muscle (Figure 5c) prevented determination of differences between conjugates in exon-skipping levels. These levels are much lower than those reported in the same TA injection model for naked PMO (8), and for exon-skipping levels in systemic mouse delivery reported for naked PMO (8,43), for RXR4XB–PMO conjugate (20) or very recently for a derivative of RXR4 (B peptide, which contains two β-Ala replacements for aminohexanoyl) (44). However, in all these studies the PMO used was a 25-mer, whereas so far only a 20-mer PNA sequence has been tested here and in our previous studies in the mouse mdx model (14). In human myoblast cell culture, 25- to 31-mer 2′OMePS ONs were found to be significantly more effective in dystrophin exon skipping than 20-mer (45), but the effect of length of ON has not been clearly evaluated for these and other ON types in vivo. Note that for the first clinical trial of a PMO ON for DMD treatment a 30-mer is being used (12). We are currently investigating the optimum length for PNA in vivo in comparison to PMO. However for either cargo type, the
significant improvements in number of dystrophin positive fibres seen in the case of Pip2a and Pip2b suggest that these peptides are good candidates for further \textit{in vivo} evaluation for enhancement of exon-skipping activity and may offer advantages over the RXR4 peptide series. Such \textit{in vivo} experiments are also currently in progress.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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