

Efficient splicing correction by PNA conjugation to an R₆-Penetratin delivery peptide

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ABSTRACT

Sequence-specific interference with the nuclear pre-mRNA splicing machinery has received increased attention as an analytical tool and for development of therapeutics. It requires sequence-specific and high affinity binding of RNaseH-incompetent DNA mimics to pre-mRNA. Peptide nucleic acids (PNA) or phosphoramidate morpholino oligonucleotides (PMO) are particularly suited as steric block oligonucleotides in this respect. However, splicing correction by PNA or PMO conjugated to cell penetrating peptides (CPP), such as Tat or Penetratin, has required high concentrations (5–10 μM) of such conjugates, unless an endosomolytic agent was added to increase escape from endocytic vesicles. We have focused on the modification of existing CPPs to search for peptides able to deliver more efficiently splice correcting PNA or PMO to the nucleus in the absence of endosomolytic agents. We describe here R₆-Penetratin (in which arginine-residues were added to the N-terminus of Penetratin) as the most active of all CPPs tested so far in a splicing correction assay in which masking of a cryptic splice site allows expression of a luciferase reporter gene. Efficient and sequence-specific correction occurs at 1 μM concentration of the R6Pen-PNA705 conjugate as monitored by luciferase luminescence and by RT-PCR. Some aspects of the R6Pen-PNA705 structure–function relationship have also been evaluated.

INTRODUCTION

A serious limitation of the use of many types of synthetic oligonucleotides (ON) and their analogues as therapeutic antisense agents has been their poor cellular delivery (1,2). Many types of vector have been designed to aid ON

delivery both for cell culture and *in vivo*. Amongst such strategies, conjugation to cell penetrating peptides (CPP) has received much recent attention (3–6).

In the case of negatively charged antisense ON, the potential of conjugated CPPs for delivery has not been realized, since there are very few publications that have shown significant biological activity (7,8). Indeed, a recent study with a well-controlled assay dealing with inhibition of *trans*-activation of the HIV-1 LTR showed some significant cell internalization of a number of CPP-ONs, but a complete lack of biological activity (9). In addition, only very modest biological activity was found for similar CPPs conjugated to synthetic short interference RNA (siRNA) targeted to a P38 MAP kinase mRNA (10).

A particularly useful HeLa cell assay for assessing the activity of CPP-ONs conjugates in a comparative manner is that established by Kole and colleagues (11) involving splice correction of an aberrant β-globin intron by 16-mer synthetic oligonucleotides (705 site) and subsequent up-regulation of firefly luciferase. This assay is straightforward to carry out and has a very high dynamic range, such that even very low activity levels can be seen as a positive luminescence read-out. CPPs conjugated to ONs that are not negatively charged, such as peptide nucleic acids (PNA) or phosphoramidate morpholino oligonucleotides (PMO) have shown significant promise in splicing correction assays and other steric block applications, for which PNA is particularly suited. For many PNA conjugates, biological activity in this and other splice alteration assays has been observed when the PNA is attached to cationic, amphipathic or other CPP peptides, but concentrations of conjugates in the 5–10 μM range almost invariably have been needed for incubation with cultured cells to see significant splice alteration activity (12–19).

Recent studies by our laboratories (19–23) and by other groups (24,25) have demonstrated that a major barrier for nuclear delivery, required for splicing correction, is the release from endocytic vesicular compartments. This was not surprising since, for polycationic CPPs such as Tat,

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Penetratin, R₉ or K₈, the vast majority of the material is internalized by an active mechanism of endocytosis, which involves electrostatic interactions with cellular heparan sulphates, and has little access to the nuclear compartment (20). Endosomolytic agents, such as chloroquine, calcium ions or high sucrose concentration (21,26), are necessary to obtain a significant splice correction activity (17–19,23), but the use of such agents *in vivo* is difficult to envisage. One possible solution is to complement the CPP with a membrane-destabilizing agent (e.g. viral fusogenic peptide or membrane-destabilizing peptide), such as has been proposed by Dowdy to improve CPP-mediated protein transfection (27), or to screen for a new peptide additive that might improve the biological activity of the CPP conjugate. In addition to the increased complexity of such a delivery system and to its cost, we have not been able to find to date a peptide or lipopeptide that showed substantially enhanced steric-block biological activity for a PNA ON conjugated to the Tat peptide (19). Likewise the co-incubation of 5 μM HA2–Penetratin fusion peptide with various CPP–PNA constructions had only a moderate effect on splice correction (18).

We, therefore, concluded that a better approach is to modify existing CPPs in order to search for peptides that may have enhanced intrinsic endosomolytic activity. Two vector strategies have been adopted, both taking into account the key roles played by Arg side chains in CPP uptake. We recently showed that (R-Ahx-R)₄–PMO705 conjugate had significant splicing correction activity in the luciferase up-regulation model at 1 μM concentration in the absence of an endosomolytic agent (28). Similarly we showed that a (R-Ahx-R)₄–PNA705 conjugate also had significant splice correction activity at 1 μM concentration (19). In parallel studies, we found substantial activity in an HIV-1 *trans*-activation inhibition assay (also requiring nuclear delivery) when a derivative of the known CPP Penetratin, in which six Arg residues were added to the N-terminus of the CPP, R₆–Penetratin (R6Pen), was disulfide-conjugated to a PNA complementary to the *trans*-activation responsive element RNA (21). We show now that this Arg-modified CPP when conjugated to a PNA targeted to the luciferase splice correction site shows by far the highest up-regulation of luciferase at both protein and RNA levels at 1 μM concentration compared to all previous CPPs studied. We also begin to characterize some aspects of the structure–function relationship and show that, for example, a W→L mutant that was reported to substantially reduce the cell penetration of Penetratin peptide (29) does not reduce the splice correction activity of the R6Pen–PNA conjugate. These results show that R6Pen might be a very good lead CPP towards further development of a suitable PNA–peptide conjugate candidate for *in vivo* studies.

MATERIALS AND METHODS

Synthesis of peptide–PNA conjugates

Synthesis of PNA. N-terminal nitropyridyl (Npys) cysteine-containing PNA oligonucleotides with additional lysine residues were synthesized on an Apex 396

Synthesizer by the Fmoc/Bhoc method as previously described (21,30) to give the general structure NH₂–Cys(NPys)–Lys–PNA–(Lys)₃–amide. PNA705 antisense is CCTCTTACCTCAGTTACA and PNA705 scrambled sequence is CTGTATACCACTTACA. Note that we have found recently that higher overall synthesis yields are obtained when the final deprotections are carried out in the absence of phenol scavenger. In some cases, N-terminal Cys-containing PNA was obtained from Panagene (www.panagen.com) and activated with dipyridyldisulfide (Pys2) as follows. To the PNA (500 nmol) was added 150 μl Pys2 (6.75 μmol, 13.5 eq.) in DMF (10 mg ml⁻¹), 15 μl 2 M triethylammonium acetate solution (pH 7) and 135 μl water. After standing for 1 h the solution was loaded on to a Sephadex NAP-10 column and eluted with 0.1% TFA solution, collecting the excluded volume. This solution was used directly in conjugation after quantification by measurement of the absorbance at 260 nm. Npys and Pys activated PNA could be used interchangeably in the conjugation reactions to form disulfide linkages.

Stably Linked K₈–PNA705 [NH₂–(Lys)₈–CCTCTTACCTCAGTTACA–Lys–amide] and Tat–PNA705 [NH₂–Gly–Arg–Lys–Lys–Arg–Arg–Gln–Arg–Arg–Arg–Pro–(O-linker)–CCTCTTACCTCAGTTACA–amide] peptide–PNA conjugates were synthesized by continuous PNA/peptide synthesis as previously described (21,30). An O-linker was added with an Fmoc-AEEA spacer (Applied Biosystems). The α-N-bromoacetyl–Lys–PNA–(Lys)₃–amide (both 705 and scrambled 705) were obtained from Panagene (Korea).

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%.

Synthesis of peptides. All peptides were prepared with free N-terminus and C-terminal amide and also contained an additional C-terminal cysteine residue to allow conjugation. Tat: GRKKRRQRRRPC, Pen: RQIKIWFQNRRMKWKKGGC and (R-Ahx-R)₄–C were obtained from Southampton Polypeptides/Activotec. R₆–Pen: RRRRRRRQIKIWFQNRRMKWKKGGC, R₆Pen_(W-L): RRRRRRRQIKILFQNRRMKWKKGGC, R₃Pen: RRRRQIKIWFQNRRMKWKKGGC and R₉Pen: RRRRRRRRRRQIKIWFQNRRMKWKKGGC were synthesized on a PerSeptive Biosystems Pioneer peptide synthesiser (100 μmol scale) using standard Fmoc/*tert*-butyl solid phase synthesis techniques as C-terminal amide peptides using NovaSyn TGR resin (Novabiochem). Deprotection of all peptides and cleavage from the solid support was achieved by treatment with trifluoroacetic acid (TFA) in the presence of triethylsilane (1%), ethane dithiol (2.5%) and water (2.5%). Purification was carried out by reversed phase HPLC as previously described (9) and analysed by MALDI-TOF mass spectrometry with the same matrix as for PNA.

Conjugation of peptides with PNA

Thioether conjugations. In a typical conjugation reaction, 50 nmol bromoacetyl PNA was dissolved in 45 μ l formamide and 10 μ l BisTris-HBr buffer (pH 7.5) and 15.6 μ l C-terminal-Cys containing peptide (8 mM, 125 nmol, 2.5 eq.) was added. The solution was heated at 40°C for 2 h and the product was purified by reversed phase HPLC at 45°C using water bath heating and analysed by MALDI-TOF mass spectrometry (Supplementary Table S1).

Disulfide conjugations. These were carried out essentially as previously described usually with a 2.5-fold excess of peptide component over PNA component. Purification was carried out by reversed phase HPLC as above and analysis by MALDI-TOF mass spectrometry (21,30)(see Supplementary Table S1).

Splice correction assay

This was carried out similarly to that described previously (28). The conjugates (Table 1) were incubated for 4 h in 1 ml OptiMEM medium with exponentially growing HeLa pLuc705 cells (1.75×10^5 cells/well seeded and cultivated overnight in 24-well plates). The conjugates were then diluted with 0.5 ml complete medium (DMEM plus 10% fetal bovine serum) and incubation continued for 20 h. Cells were washed twice with ice-cold PBS and lysed with Reporter Lysis Buffer (Promega, Madison, WI, USA). Luciferase activity was quantified with a Berthold Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany) using the Luciferase Assay System substrate (Promega, Madison, WI, USA). Cellular protein concentrations were measured with the BCATM Protein Assay Kit (Pierce, Rockford, IL, USA) and read using an ELISA plate reader (Dynatech MR 5000, Dynatech Labs, Chantilly, VA, USA) at 550 nm. Levels of luciferase expression are shown as relative light units (RLUs) per microgram protein. All experiments were performed in triplicate. Each data point was averaged over the three replicates.

Cell permeability assay (Flow cytometry analysis)

To analyse the cell permeabilization of CPP-PNA conjugates, exponentially growing HeLa pLuc705 cells (3×10^5 cells seeded and grown overnight in 30 mm plates) were incubated for 4 h with the CPP-PNA705 conjugates at different concentrations. The cells were then washed twice with PBS, detached by incubating with trypsin for 5 min at 37°C (0.5 mg ml⁻¹)/EDTA.4Na (0.35 mM), and washed by centrifugation (5 min, 900 \times g) in ice-cold PBS containing 5% FCS. The cell pellet was resuspended in ice-cold PBS containing 0.5% FCS and 0.05 μ g/ml propidium iodide (PI) (Molecular Probes, Eugene, OR, USA). Fluorescence analysis was performed with a BD FacsCanto flow cytometer (BD Biosciences, San Jose, CA, USA). A minimum of 20 000 events per sample were analysed.

RT-PCR analysis of splice correction

HeLa pLuc705 cells were plated at 30 000 cells/well in a 24-well plate 24 h before treatment. After overnight incubation, the cells were washed with PBS and incubated in 1 ml OptiMEM containing 1 μ M of the indicated conjugates (naked PNA705, Pen-s-s-PNA705, R6Pen-s-s-PNA705, R6Pen-s-s-PNA705scr or R6Pen(W-L)-s-s-PNA705) for 4 h and the conjugates were then diluted with 0.5 ml of DMEM containing 10% FCS and allowed to grow for 20 h. Cells were then washed twice with PBS. Total RNA was extracted from the cells using the High pure RNA isolation Kit (Roche Applied Science). The extracted RNA was examined by RT-PCR (MJ Research PTC200 Peltier Thermal cycler) with forward primer 5'TTG ATA TGT GGA TTT CGA GTC GTC3' and reverse primer 5'TGT CAA TCA GAG TGC TTT TGG CG3'. The products were analysed on a 2% agarose gel (Figure 7A).

For dose-dependence experiments (Figure 7B), cells were treated as described above with increasing concentrations of R6Pen-s-s-PNA705 or R6Pen-PNA705 conjugates. After carrying out the luciferase assay and

Table 1. Sequences and nomenclature of CPP-PNA705 conjugates

Name	Sequences ^a
	Stably linked
R6Pen-PNA705 (S-CH2) ^b	NH ₂ -RRRRRRRQIKIWFQRRMKWKKGGC-thioacetyl-K-CCT CTT ACC TCA GTT ACA-KKK-amide
R6Pen-PNA705scr (S-CH2) ^b	NH ₂ -RRRRRRRQIKIWFQRRMKWKKGGC-thioacetyl-K-CCT GTT ATA CCA CTT ACA-KKK-amide
Tat-PNA705 ^c	NH ₂ -GRKKRRQRRP-O linker-CCT CTT ACC TCA GTT ACA-amide
K8-PNA705 ^c	NH ₂ -KKKKKKKK-CCT CTT ACC TCA GTT ACA -K-amide
(R-Ahx-R)4-PNA705(S-CH2) ^b	NH ₂ -R-Ahx-RR-Ahx-RR-Ahx-RR-Ahx-R-C-thioacetyl-K-CCT GTT ATA CCA CTT ACA-KKK-amide
	Disulfide linked ^d
Pen-s-s-PNA705	NH ₂ -RQIKIWFQRRMKWKKGGC-ss-CK-CCT CTT ACC TCA GTT ACA-KKK-amide
R6Pen-s-s-PNA705	NH ₂ -RRRRRRRQIKIWFQRRMKWKKGGC-ss-CK-CCT CTT ACC TCA GTT ACA-KKK-amide
R6Pen-s-s-PNA705scr	NH ₂ -RRRRRRRQIKIWFQRRMKWKKGGC-ss-CK-CCT GTT ATA CCA CTT ACA-KKK-amide
R6Pen(W-L)-s-s-PNA705	NH ₂ -RRRRRRRQIKILFQRRMKWKKGGC-ss-CK-CCT CTT ACC TCA GTT ACA-KKK-amide
R3Pen-s-s-PNA705	NH ₂ -RRRRQIKIWFQRRMKWKKGGC-ss-CK-CCT CTT ACC TCA GTT ACA-KKK-amide
R9Pen-s-s-PNA705	NH ₂ -RRRRRRRRRQIKIWFQRRMKWKKGGC-ss-CK-CCT CTT ACC TCA GTT ACA-KKK-amide

^aBold denotes amino acid residues, normal typeface for PNA residues and an underlined residue shows a W to L mutation in Penetratin.

^bA thioacetyl linker is formed between a C-terminal cysteine on the peptide and a N- α -bromoacetyl-substituted PNA.

^cA continuous synthesis (an O-linker is added with an Fmoc-AEEA spacer monomer from Applied Biosystems).

^dAll disulfide-linked conjugates are formed between a C-terminal Cys residue on the peptide and an N-terminal Cys residue on the PNA part.

BCA™ Protein Assay, the remaining cell lysates (about 270 μ l) 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 iso-propanol for RNA precipitation was increased to give a 1:1 mixture with the aqueous phase. The RT-PCR was carried out as described above and agarose gels were scanned using Gene Tools Analysis Software (SynGene, Cambridge, UK).

RESULTS

Figure 1 shows a comparison of the splice correction activities at 1 μ M concentration of unconjugated PNA705, K8-PNA705 and Tat-PNA705, the activity of each of which is known to be chloroquine-dependent (9,17,18,23), together with R6Pen-PNA705 and (R-Ahx-R)₄-PNA705 (19) in the absence of an endosomolytic agent. In all cases, PNAs were conjugated to the carrier peptides through stable amide or thioacetyl linkages (Table 1 for construct details). R6Pen conjugate, and to a lesser extent (R-Ahx-R)₄ conjugate, gave rise to a strong up-regulation of luciferase under conditions where K8 and Tat peptide conjugates were essentially inactive. Note that the scale of light units is shown in relative light units per microgram protein, demonstrating the very high level of activity seen for R6Pen-PNA705. The low level of activity for Tat-PNA705 agrees with results recently reported by two other laboratories, where similarly low splice correction was seen also for Penetratin, R₉ and Transportan at 1 μ M concentration (17,18) and only at 5–10 μ M concentrations did some conjugates (notably Transportan) show significant splice correction activity. Thus, R6Pen appears substantially more effective as a CPP and leads to much stronger splice correction activity compared to our previously used (R-Ahx-R)₄-PNA705.

The splice correction activity of the R6Pen conjugate is sequence-specific, since no splice correction activity is seen when this CPP vector is conjugated to a scrambled version of PNA705 (Figure 2). Note that luciferase activity levels vary somewhat between experiments as pointed out by Bendifallah *et al.* (17). Normalization of the data to the basal luciferase expression in untreated cells, as proposed by these authors, gives rise to much less apparent variation between experiments (see Supplementary Data, Figures 1 and 2), but we have chosen here to show un-normalized values just to demonstrate the high activity levels.

To characterize further the properties of the R6Pen-PNA705 conjugate, we monitored the dose-dependence of splice correction, as measured by luciferase up-regulation, at concentrations between 0.1 and 2.5 μ M (Figure 3). R6Pen-PNA705 allows an efficient dose-dependent splice correction activity in the absence of chloroquine (Figure 3, white bars) under conditions where no toxicity was seen, as judged by measurement of PI uptake by flow cytometry (Supplementary Data, Figure 3). The proportion of permeabilized cells remained <3% as compared to the

untreated controls in cells incubated with the various CPP-PNA conjugates at 1 μ M (e.g. at the concentration allowing almost complete splicing correction). The addition of chloroquine improved the splice correction activity, which demonstrates that some of the conjugate still remains entrapped in endosomal compartments in keeping with an endocytotic mechanism of cell uptake. However, the incremental improvement in splice correction activity afforded by chloroquine addition was somewhat smaller at the higher concentrations (approximately 2- to 3-fold, Figure 3, grey bars), than those we obtained previously with K8 and Tat conjugates of PNA or PMO, where a 10-fold increase or more was often observed (19,23).

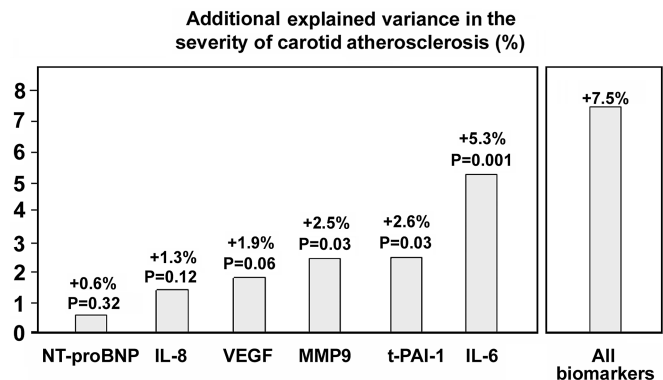


Figure 1. Comparison of splice correction efficiencies by various CPP-PNA705 conjugates. HeLa pLuc705 cells were incubated for 4 h in OptiMEM in the absence (control), in the presence of 1 μ M PNA705 alone, or in the presence of 1 μ M CPP-PNA705 conjugates. Luciferase expression was quantified 20 h later and was expressed as RLU per microgram protein. Each experiment was made in triplicate and error bars (standard deviations) are indicated.

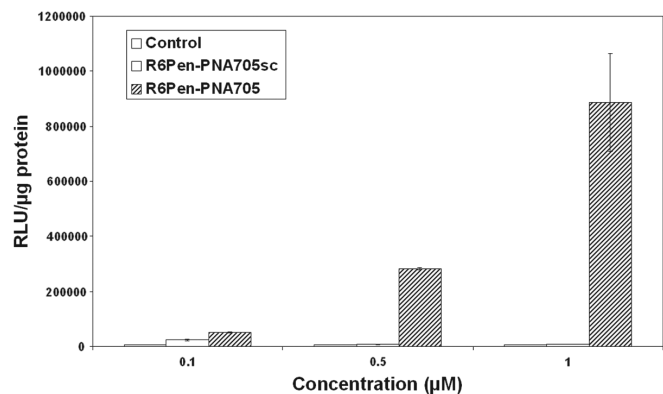


Figure 2. Splice correction specificity. HeLa pLuc705 cells were incubated for 4 h in OptiMEM in the absence (control) of correcting PNA, in the presence of the stably linked R₆Pen-PNA705 splice correcting conjugate, or in the presence of its scrambled version at the indicated concentrations. Luciferase expression was quantified 20 h later and was expressed as RLU per microgram protein. Each experiment was made in triplicate and error bars (standard deviations) are indicated.

We next investigated the importance of the stability of the linkage between the delivery peptide and the PNA cargo. It has been suggested by others that if a disulfide-linked conjugate is able to escape from the endocytic compartments and reaches the cytosol, the disulfide bridge might be reduced, thus allowing free PNA to be released (31). A new conjugate R6Pen-s-s-PNA705 (Table 1) was therefore constructed with a linker containing a disulfide bridge, similar to that which we have previously used in studies of HIV-1 Tat-dependent *trans*-activation inhibition (21). This conjugate was tested in the splice-correction assay in parallel with the stably linked R6Pen-PNA705 and indeed showed a slightly (but reproducibly) higher activity (Figure 4). However, the relatively small difference demonstrates that the nature of the linkage is not a principal factor governing splice correction activity. Nevertheless, we decided to use the more active disulfide-bridged conjugates for further studies on the structure-function relationship.

In order to determine the effect of the N-terminal Arg stretch on splice correction activity, we constructed a series of R(z)Pen-s-s-PNA705 conjugates with $z \in \{0, 3, 6 \text{ and } 9\}$. These R(z)Pen-s-s-PNA705 conjugates were tested at 0.5 and 1 μM in the splice correction assay in the absence of chloroquine (Figure 5). Pen-s-s-PNA705 at 1 μM displays only a very weak activity, consistent with previous results of others (17,18). The activity level is strongly enhanced by the addition of an Arg tail by factors of 16, 43 and 28 for $z = 3, 6$ and 9, respectively. Thus, at 1 μM concentration, the optimum activity is obtained for R₆. No significant differences were seen in cell toxicity for any of the conjugates at this concentration as judged by flow cytometry and PI uptake (Supplementary Data, Figure 3).

Previous studies (29) have shown that the substitution of the tryptophan residues that occurs naturally in the *Antennapedia* homeodomain helix 3 sequence by a leucine residue decreased the cell internalization of

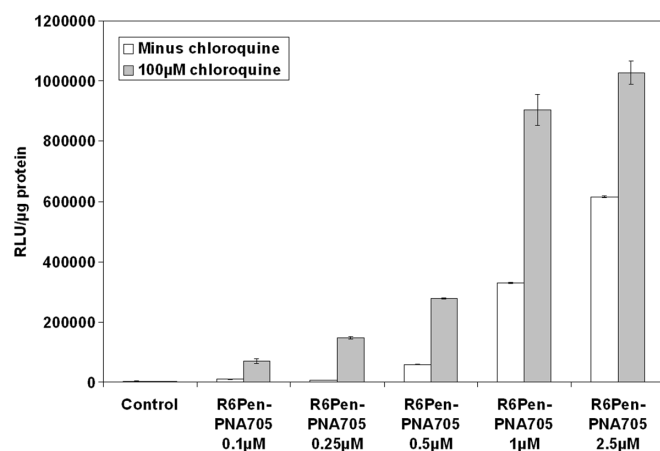


Figure 3. The effect of chloroquine on splice correction. HeLa pLuc705 cells were incubated for 4 h in OptiMEM with R₆Pen-PNA705 correcting conjugates at the indicated concentrations in the absence (white bars) or in the presence (grey bars) of 100 μM chloroquine. Luciferase expression was quantified 20 h later and was expressed as RLU per microgram protein. Each experiment was made in triplicate and error bars (standard deviations) are indicated.

Penetratin peptide. Surprisingly, the R6Pen(W-L)-s-s-PNA705 conjugate displayed a slightly higher splicing correction activity than the unmodified R6Pen-PNA705 (Figure 6). This indicates that the Penetratin part of the R6Pen-PNA conjugate has a completely different effect in enhancement of membrane permeabilization when it is located within the PNA conjugate context as compared to the Penetratin peptide alone.

In most studies using the HeLa-pLuc705 model, splice-correction is monitored by the quantification of luciferase luminescence activity (17–19,22,23,28). However, this assay gives only a relative appreciation of splice correction activity between different conjugates. In contrast, use of RT-PCR allows the evaluation of the completeness of splice correction by comparison of the

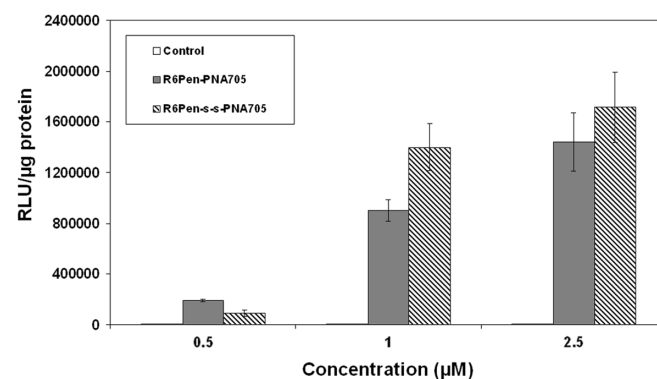


Figure 4. The effect of CPP-PNA705 linker stability on splice correction. HeLa pLuc705 cells were incubated for 4 h in OptiMEM in the absence (control) or in the presence of R₆Pen-PNA705 conjugates at the indicated concentrations. The R₆Pen and PNA705 moieties were conjugated by a stable thioacetyl or by a reducible disulfide linker. Luciferase expression was quantified 20 h later and was expressed as RLU per μg protein. Each experiment was made in triplicate and error bars (standard deviations) are indicated.

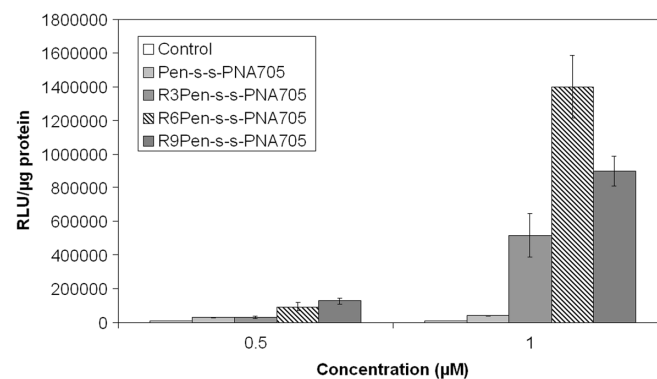


Figure 5. The effect of the number of arginine residues on splice correction. HeLa pLuc705 cells were incubated for 4 h in OptiMEM in the absence (control) or in the presence of R_zPen-PNA705 conjugates (with $z = 0, 3, 6$ or 9) at the indicated concentrations. Luciferase expression was quantified 20 h later and was expressed as RLU per microgram protein. Each experiment was made in triplicate and error bars (standard deviations) are indicated.

amounts of uncorrected and corrected mRNA, as has been used with this splice correction assay for cationic lipid-based transfection methods (11,32). We, therefore, carried out RT-PCR on RNA samples extracted from HeLa-pLuc705 cells incubated with various conjugates (Figure 7A). As expected, no RT-PCR products corresponding to the correctly spliced mRNA were detected in cells treated with 1 μ M of free PNA705, Pen-s-s-PNA705, or scrambled control R6Pen-s-s-PNA705sc, as seen in lanes 1, 2 and 3, respectively. In contrast, a very high proportion of correctly spliced mRNA was found in cells treated with 1 μ M R6Pen-s-s-PNA705 (lane 4) or with R6Pen(W-L)-s-s-PNA705 (lane 6).

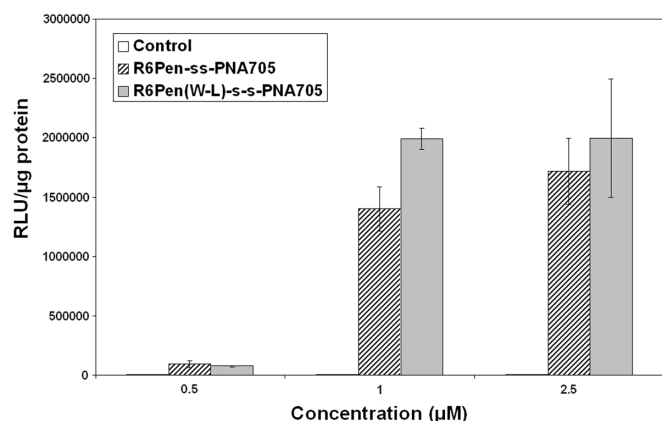


Figure 6. The effect of a W→L Penetratin mutation on splice correction. HeLa pLuc705 cells were incubated for 4 h in OptiMEM in the absence (control) or in the presence of CPP-PNA705 conjugates at the indicated concentrations. Luciferase expression was quantified 20 h later and was expressed as RLU per microgram protein. Each experiment was made in triplicate and error bars (standard deviations) are indicated.

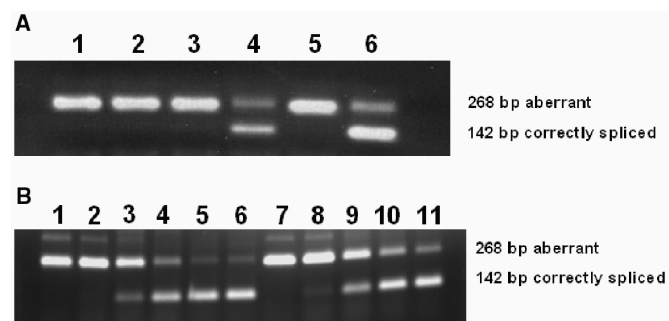


Figure 7. RT-PCR analysis of splice correction. (A) HeLa pLuc705 cells were incubated for 4 h in OptiMEM in the absence (control), in the presence of 1 μ M PNA705 alone or in the presence of 1 μ M CPP-PNA705 conjugates. Total RNA was extracted 20 h later and amplified by RT-PCR. PCR products from incorrectly (268 bp) and correctly (142 bp) spliced luciferase pre-mRNA were analysed on a 2% agarose gel. Lane 1: control, Lane 2: PNA705 alone, Lane 3: Pen-ss-PNA705, Lane 4: R₆Pen-s-s-PNA705, Lane 5: R₆Pen-s-s-scrambled PNA705, Lane 6: R₆Pen(W-L)-s-s-PNA705. (B) Dose dependencies of splice correction using 1 μ g of total RNA extracted, amplified by RT-PCR and analysed as in (a). Lane 1: control of untreated cells, Lanes 2–6: cells treated with 0.25, 0.5, 1, 2 or 4 μ M R6Pen-s-s-PNA705 respectively and Lanes 7–11: cells treated with 0.25, 0.5, 1, 2 or 4 μ M stably linked R6Pen-PNA705, respectively.

The dose-dependences of splice correction for R6Pen-s-s-PNA706 and stably linked R6Pen-PNA705 were assessed by the RT-PCR assay (Figure 7B). The EC₅₀s of splice correction at the RNA level were estimated as 0.7 \pm 0.3 μ M and 1.0 \pm 0.3 μ M, respectively. EC₅₀s were also estimated from the amounts of conjugate required to raise the luciferase luminescence levels to 50% of the observed plateau values (data not shown). These values were found to be 0.9 \pm 0.2 μ M and 1.0 \pm 0.2 μ M, respectively.

DISCUSSION

The nuclear delivery of steric-block ON analogues conjugated with most CPPs for splice correction or exon skipping has been hampered by endosome trapping, unless an endosome disturbing drug or peptide is added, or high CPP-PNA conjugate concentrations are used. Bearing in mind the key role played by cationic amino acids for CPP uptake, we have appended varying numbers of arginine residues to the N-terminal end of Penetratin, a CPP which by itself does not impart on the PNA a significant amount of splice correction ability. R6Pen turned out to be the most active. The level of activity obtained for splice correcting conjugated PNA is higher than for all other CPPs tested to date, including the recently described (R-Ahx-R)₄ vector (19,28). Remarkably, R6Pen-PNA705 conjugates are highly active at 1 μ M concentrations in the absence of any endosomolytic agents.

Quantification of luciferase expression, as carried out here and also in most published work to date, is a sensitive and convenient assay, which allows one to compare several conjugates quickly in terms of efficiency or specificity, and is thus the method of choice for structure-activity relationships studies. However, such data are expressed in relative light units and do not allow direct determination of the extent to which aberrant splicing has been corrected. RT-PCR products from the aberrantly and correctly spliced luciferase pre-mRNA can be separated easily by agarose gel electrophoresis, thus allowing evaluation of the extent of splice correction under various conditions. RT-PCR data closely parallel luciferase luminescence measurements and indicate that the R6Pen-ss-PNA705 and the W→L variant allow sequence-specific splicing correction at 1 μ M concentration to a high level (about 60–70%), whilst PNA705 alone or Pen-s-s-PNA705 are totally inactive. The levels of activity we have obtained (EC₅₀ of 0.7–1.0 μ M) now start to approach those obtained with the same assay by cationic lipid transfection using leashed PNA or other modified ON types (11,32).

The achievement of a fair proportion of correction at low conjugate concentration is a key issue in the development of steric block ONs as potential therapeutics. By use of PI as an index of membrane permeabilization, we have indeed verified that R6Pen did not perturb membrane integrity of HeLa cells at the active dosage. Previous studies from our group have established that high (>5 μ M) concentrations of CPP-ON as R₉ or K₈-ON

led to significant increase of PI uptake thus precluding further developments (23).

We have no explanation at this stage for the dramatically increased splice correction activity of R6Pen as compared to Pen or as compared to several Arg-rich CPPs. It is worth emphasizing in this respect that the W→L mutation in the Penetratin moiety, which is known to inhibit Penetratin peptide uptake (29), does not affect splice correction by R6Pen-PNA705 and instead gave rise to a slightly higher activity (Figures 6 and 7), thus inferring different mechanisms by which this CPP operates. Along the same lines, chloroquine has a significantly lower effect on splice correction by R6Pen-PNA705 (Figure 3) as compared to Tat-PNA705 (19) or K8-PNA705 (23), in keeping with its improved intrinsic endosomal escape. We are also able to rule out significant effects of the Lys residues on the PNA part on splice correction activity. Indeed we have found recently that R6Pen disulfide linked to a PNA 18-mer containing just one Lys residue on each end behaved identically to the corresponding conjugate containing four Lys residues (data not shown). Further mechanistic studies are in progress, but it should be noted that we have deliberately avoided on these conjugates the use of fluorescent labels, which are commonly used to track cellular uptake by confocal microscopy. Such labels alter the hydrophobicity of the conjugate at a particular region. This may alter the ability of the PNA-peptide to be released from endosomal compartments. Concerns about this have emerged recently in the case of our parallel studies on inhibition of HIV-1 Tat-dependent *trans*-activation (21). We have been unable so far to construct a conjugate that contains a fluorescein label on the PNA part of a R₆-Penetratin-PNA conjugate targeted to TAR without losing all intra-nuclear inhibition activity in the absence of chloroquine in our HeLa cell assay (Turner, J.J., Arzumanov, A.A., Ivanova, G.D. and Gait, M.J., unpublished results). Further, there does not appear to be a strong correlation of the amount of fluorescent oligonucleotide reagent seen to be taken up by cells and their biological activity (21,23,25,28), as has also become apparent in the design of lipid-based reagents for delivery of siRNA (33). Thus, more sophisticated ways of tracking locations of nucleic acids-based reagents and determining the precise compartments where activity takes place will be needed before such types of experiment become fully meaningful.

Whether CPP delivery peptides and their cargoes should be conjugated through stable or unstable linkers has often been debated, but few direct comparisons have been provided. In our case, a disulfide-linked conjugate was slightly (but reproducibly) more active than a stably conjugated PNA. Thus, we are now in the process of carrying out further more detailed structure-function analyses using such disulfide linkers to try to understand how the various parts of the R6-Penetratin peptide contribute to obtaining intra-nuclear splice correction activity. The disulfide linker strategy may also be less susceptible to problems arising from steric interference by the conjugated delivery vehicle, or from potential non-specific binding of the vector to non-targeted entities. However, use of PNA-peptide conjugates *in vivo* may

require a more stable linkage and our work shows that a thioacetyl linker is also compatible with high-level splice correction activity.

The fact that strong splicing correction (as judged by the RT-PCR analysis) can be achieved at much lower (1 μM) concentration of the correcting ON than has previously proved possible opens up promising perspectives for *in vivo* applications. We hope that further optimization of the peptide-PNA construct will lead to a construct suitable for *in vivo* studies, and eventually for instance towards the treatment of disease-associated splicing defects [cancer, thalassemia, etc. (34)] or in exon-skipping strategies, as are now being considered for the treatment of Duchenne muscular dystrophy (35,36).

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

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