Targeted gene correction using psoralen, chlorambucil and camptothecin conjugates of triplex forming peptide nucleic acid (PNA)

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Abbreviations: PNA, peptide nucleic acids; TFO, triplex-forming oligonucleotide; ZFN, zinc finger nuclease; ICL, interstrand cross-linking; ssODN, single stranded oligonucleotide; SFHR, short fragment homologous repair; bp, base pair; FACS, fluorescence-activated cell sorting; topo I, topoisomerase I; kb, kilo base

Gene correction activation effects of a small series of triplex forming peptide nucleic acid (PNA) covalently conjugated to the DNA interacting ligands psoralen, chlorambucil and camptothecin targeted proximal to a stop codon mutation in an EGFP reporter gene were studied. A 15-mer homopyrimidine PNA conjugated to the topoisomerase I inhibitor camptothecin was found to increase the frequency of repair domain mediated gene correctional events of the EGFP reporter in an in vitro HeLa cell nuclear extract assay, whereas PNA psoralen or chlorambucil conjugates both of which form covalent and also interstrand crosslinked adducts with dsDNA dramatically decreased the frequency of targeted repair/correction. The PNA conjugates were also studied in mammalian cell lines upon transfection of PNA bound EGFP reporter vector and scoring repair of the EGFP gene by FACS analysis of functional EGFP expression. Consistent with the extract experiments, treatment with adduct forming PNA conjugates (psoralen and chlorambucil) resulted in a decrease in background correction frequencies in transiently transfected cells, whereas unmodified PNA or the PNA-camptothecin conjugate had little or no effect. These results suggest that simple triplex forming PNA conjugates have little effect on proximal gene correctional events whereas PNA conjugates capable of forming DNA adducts and interstrand crosslinks are strong inhibitors. Most interestingly the PNA conjugated to the topoisomerase inhibitor, camptothecin enhanced repair in nuclear extract. Thus the effects and use of camptothecin conjugates in gene targeted repair merit further studies.

Introduction

The growing interest in targeted genomic therapy has increased the search for new and more efficient sequence targeting molecules. Such molecules can be exploited to promote homologous recombination or other sequence directed repair between a specific targeted gene segment and an externally supplied gene correction DNA donor or the insertion of externally supplied genetic material at a specified target site in the genome.1,2 Two of the more promising gene targeting molecules are designed zinc-finger nucleases (ZFNs) and triplex-forming oligonucleotides (TFOs). Indeed using gene targeted ZFNs levels of targeted genetic editing of close to 40% have recently been obtained under optimized conditions in specific cell types.3 TFOs belong to an alternative class of targeting molecules that form triple helices via Hoogsteen base pairing in the major groove of the DNA double helix at homopyrimidine/homopurine tracts.4 Despite the success of ZFN assisted gene repair, TFOs may eventually present a valuable clinical alternative because of possible deleterious side effects from off target ZFN-induced DNA double strand breaks, stable insertion of ZFN producing genes in the genome, ZFN related cytotoxicity, immunogenic responses and poor regulation of ZFN cleavage rates.5 Several studies have shown that gene correctional activities can be augmented by simply introducing triple-helix complexes in the vicinity of the gene correction target.6 Furthermore, TFOs can be conjugated to a broad spectrum of DNA-interacting ligands, which may further activate and/or recruit the cellular DNA repair enzymes (e.g., for homologous recombination). Since natural, unmodified oligonucleotides are susceptible to nuclease degradation, several analogues and chemically modified TFOs have been developed, ensuring higher in vivo stability.4 Peptide nucleic acids (PNAs) which constitute a particular sub-group of triplex forming oligomers, are uncharged DNA mimics, in which a pseudo-peptide-linked backbone replaces the normal deoxyribose phosphodiester structure of DNA (Fig. 1). This replacement averts intracellular nuclease and protease digestion of the oligomer, and enhances the hybridization of PNA to DNA strands, as there is no electrostatic repulsion between the two backbones.7

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previous studies on PNA oligomers in gene correction approaches have focused on DNA helix invading bis-PNAs,8,9 that form P-loops with an internal PNA-DNA-PNA triplex binding to the target DNA strand,10 or pseudocomplementary PNA forming a complementary double helix exhibiting a highly inhibitory effect on gene correction.6 Therefore, we synthesised two PNA oligomers conjugated to psoralen or chlorambucil (Fig. 2), respectively. Psoralen can induce thymine-thymine dsDNA interstrand cross-links at TA sites upon photo-activation (UVA irradiation), while chlorambucil readily forms guanine-guanine cross-links by alkylation. Third strand-conjugated chlorambucil groups do, however, mainly form mono-adducts with DNA helices.24 To analyse if targeted single-strand breaks in DNA would influence local gene correction events, we also synthesised a camptothecin-conjugated PNA oligomer. Camptothecin can interact with intermediate topoiso- merase I/DNA complexes following topoiso- merase I nicking of DNA. This stabilizes the topo I/DNA complex, thereby preventing the resealing of the nicked DNA strand. Such complexes are not tolerated, and are subject to cellular protease degradation, which can lead to the formation of DNA single strand breaks.26 Previously, TFO-camptothecin conjugates have been shown to promote DNA strand break in vitro.25 PNA oligomers were incubated with the vector, photo-activated (UVA irradiation) when necessary, and mixed with the appropriate DNA donor before incubation in mammalian cell nuclear extract and in a cell culture, extra-chromosomal assay. In contrast, a conventional triplex forming PNA (TFP) showed little or no effect on gene correction, whereas a PNA-camptothecin conjugate was found to stimulate gene correction events in mammalian cells.

**Results**

**Design of vector based assay for quantification of targeted gene correction.** Numerous previous studies have shown that gene correction in human cells in vitro can be performed using corrective DNA donor fragments.1,20,21 Thus, we designed a vector-based acceptor gene assay in conjunction with sequence specific DNA donor repair fragments. The assay comprises a closed circular target vector (P25) and a single stranded oligonucleotide or a double stranded PCR derived DNA donor fragment (Fig. 2). The P25 vector contains a mutated EGFP gene in which codon 27 has been converted from AAG into the translational termination codon TAG. P25 is the target for gene correction activity, where restoration of the AAG codon can be detected by the expression of functional fluorescent EGFP in either bacteria or in mammalian cells (Fig. 2). A target site, which has been shown to support the formation of stable PNA triplexes,25 was inserted upstream of the mutated EGFP gene (150 bases between this site and the point mutated codon 27). The efficiency of sequence restoration (TAG to AAG) was studied using two different gene repair domains, a single-stranded 53-mer oligonucleotide (ssODN #95) or a 1.65 kb double stranded PCR DNA fragment (SxP34) (aiming at exploiting the short fragment homologous repair (SFHR) donorn approach).1,21 In order to explore the effect of various DNA interacting ligands targeted by a PNA, four 15mer PNA conjugates were synthesised in which the “natural” cytosine bases of the PNA were replaced by pseudo-isocytosines (Fig. 2) this abolishes the requirement for acidic conditions to obtain efficient triplex binding.22,23 Previous reports on cellular gene correction have shown that triplex forming oligonucleotides conjugated to an inter-strand cross-linker (ICL) can increase the frequency of correction.6 Therefore, we synthesised two PNA oligomers conjugated to psoralen or chlorambucil (Fig. 2, respectively). Psoralen can induce thymine-thymine dsDNA interstrand cross-links at TA sites upon photo-activation (UVA irradiation), while chlorambucil readily forms guanine-guanine cross-links by alkylation. Third strand-conjugated chlorambucil groups do, however, mainly form mono-adducts with DNA helices.24 To analyse if targeted single-strand breaks in DNA would influence local gene correction events, we also synthesised a camptothecin-conjugated PNA oligomer. Camptothecin can interact with intermediate topoiso- merase I/DNA complexes following topoiso- merase I nicking of DNA. This stabilizes the topo I/DNA complex, thereby preventing the resealing of the nicked DNA strand. Such complexes are not tolerated, and are subject to cellular protease degradation, which can lead to the formation of DNA single strand breaks.26 Previously, TFO-camptothecin conjugates have been shown to promote DNA strand break in vitro.25 PNA oligomers were incubated with the vector, photo-activated (UVA irradiation) when necessary, and mixed with the appropriate DNA donor before incubation in mammalian cell nuclear extract or co-transfection into mammalian cells.

**Effects of triplex forming PNA oligomers on gene correction in nuclear extracts.** An in vitro HeLa nuclear extract was used to evaluate the effect of the PNA oligomers on targeted gene correction. The nuclear extract was thoroughly characterized in terms of the activity of essential DNA repair systems (See Sup.Material). Non-homologous end joining (NHEJ) activity was demonstrated by introducing linearised dsDNA fragments with defined ends. Four different combinations of defined ends were used, which ranged from blunt ends to 4 base overhangs. The circularisation of fragments was taken as evidence for NHEJ activity. Nucleotide
binding of PNA3051 to the labeled fragment (Fig. 3) was demonstrated by mobility shift upon PNA addition. In contrast, formation of triplex invasion complexes was not observed under these incubation conditions. Formation of PNA triplexes at equivalent concentrations was observed for all tested PNA oligomers (data not shown).

Following extract incubation, quantification of gene correction was performed by transforming purified DNA material from extract incubations into *E. coli*. Subsequently, the fraction of fluorescent bacterial cells was quantified by FACS analysis (Fig. 4). Consistent with results from previous reports exploiting similar gene correction assays, 26,27 co-incubation of only P25 and #95 (ssODN technique) resulted in easily detectable levels (19 x 10^-5) of gene correction (Fig. 5 and white bar). Target binding of PNA3051 (no conjugate) induced a minor (but significant, p < 0.01) increase in the gene correction rate, compared to the standard ssODN reaction (white bar), suggesting that PNA triplex formation alone can augment gene correctional activity.

excision repair (NER) activity was demonstrated by incubating UV-treated (254 nm) P25 plasmid in the extract, and incorporation of supplemented α-32P-dATP was taken as evidence for NER activity. Homologous recombination (HR) activity was demonstrated using a restriction enzyme treated P25 vector, where a 280 bp segment of the EGFP gene had been removed from the vector. The extract co-incubation of this fragmented vector with a PCR DNA donor fragment containing full EGFP gene sequence resulted in circularised vectors expressing functional EGFP in bacteria. To assess transcriptional activity, P25 was co-incubated with a NTP-mix containing α-32P-GTP in the extract. This resulted in clear levels of labelled RNA products as analyzed by gel electrophoresis, thereby showing that the extract possesses transcriptional activity.

Finally, to assess PNA triplex formation at the target site, gel shift assays were performed. A labelled 150 bp double stranded fragment containing the relevant target binding site was incubated with increasing amounts of PNA oligomers. Specific triplex binding of PNA3051 to the labeled fragment (Fig. 3) was demonstrated by mobility shift upon PNA addition. In contrast, formation of triplex invasion complexes was not observed under these incubation conditions. Formation of PNA triplexes at equivalent concentrations was observed for all tested PNA oligomers (data not shown).

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Figure 2. Experimental vector system. (A) The target vector (p25), containing a target site for triplex binding upstream to a point mutated EGFP gene. The point mutation can be corrected using one of two DNA donor fragments (#95 or SxP34). (B) PNA oligomers. T, thymine; J, pseudoisocytosine; Lys, lysine; DMLys, dimethyllysine; eg1, ethyleneglycol linker.
Additionally, in order to analyse if the effect from the camptothecin-PNA were connected to camptothecin interaction with topo I/DNA complexes, extracts were supplemented with ubiquitin. Permanently stalled topo I/DNA complexes are tagged with ubiquitin for proteasomal degradation. Thus, an increased concentration of ubiquitin could increase the rate of protein/DNA complex processing, thereby increasing the gene correction rate. Also, ubiquitin serves as modulator of several DNA repair mechanisms and consequently, increased concentrations of ubiquitin could increase the processing rates for these reactions. However, if ubiquitin stimulates DNA repair processing in general, stimulation of gene correction rates should be observed using any of the present PNA oligomers. From the data presented in Figure 7 it can be seen that ubiquitin supplementation significantly augmented the stimulatory effect of the camptothecin-PNA. This was observed using either of the correction assays. In contrast, no significant effects on correction rates were observed using no PNA, unmodified, psoralen-conjugated or chlorambucil-conjugated PNAs (data not shown). This indicates that ubiquitin signalling is specifically connected to the significant stimulation on gene correction using camptothecin-PNAs, most likely through an increased proteasome removal of stalled topo I/DNA complexes.

Effects of triplex PNAs in an ex vivo cellular assay. In order to analyze whether the observed effects were specific for the ssODN assay, an alternative assay setup was employed. This SFHR assay utilized a PCR derived 1.65 kb double stranded donor DNA fragment (SxP34, Fig. 2), which resembles gene modification assays that have previously been directed at epigenomical or chromosomal targets. The 1.65 kb SxP34 donor fragment was found to be optimal, from experiments applying dsDNA donors ranging from 300 bp to 2,500 bp (data not shown). The results presented in Figure 6 show that the effect of switching from the ssODN assay (Fig. 5) to SFHR essentially did not change the relative effect of the individual PNA oligomers, except that the un conjugated PNA3051 did not show any significant effect on gene correction rates in this assay. As in the ssODN assay, PNA oligomers that can react covalently with the DNA helix (photo-activated PNA3084 and PNA3349) significantly reduced the gene correction to near background rates, and the camptothecin-linked PNA (PNA3250) increased the correctional frequency 2-fold relative to the non-stimulated SFHR correction frequency (white bar).

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augment the correction frequency. PNAs that can react covalently with the DNA helix (photo-activated psoralen-conjugated or chlorambucil-conjugated) clearly caused dramatic inhibition of gene correction, as also seen in the in vitro extract assays. Indeed, photo-activated psoralen-PNA (PNA3084) triplexes reduced the frequency of correction to approximately 2% of that of non-stimulated SFHR directed correction (white bar). HeLa cells have been reported to be refractory in repairing psoralen-linked triplex forming oligonucleotides. Since this finding is in accordance with the present observation on gene correction activities, we decided to perform a parallel analysis in DC-F3 cells. As can be observed from the data presented in Figure 10, the decrease in correctional activity is not limited to HeLa cells. Even though the absolute gene correction frequencies are ~10-fold higher in DC-F3 cells compared to HeLa cells (Figs. 9 and 10), the relative correction frequencies are essentially identical in the two cell lines. Additional experiments using Att-20 and MCF-7 cell lines gave similar results (not shown). In contrast to the results from the SFHR in vitro nuclear extract assay (Fig. 4), the camptothecin-PNA (PNA3250) did not appear to have an effect on correctional events in either cell line. Possible causes for the divergent results in nuclear extracts and mammalian cells using camptothecin-PNA will be discussed below.

This study has demonstrated that PNA oligomers, which can react covalently with the DNA helix, appear to impede gene correction in in vitro nuclear extract and in cell culture, and that camptothecin-PNA oligomers can significantly increase targeted gene correction activity in nuclear extracts. The inhibitory effect of (light activated) psoralen-PNA and chlorambucil-PNA conjugates in extracts was independent of whether the corrective donor DNA fragment was a 53 base single stranded oligonucleotide or a 1.65 kb double stranded PCR fragment. As these donors are expected to exchange sequence information with the target vector via different recombination/repair mechanisms, the impeding effect from especially the psoralen-PNA conjugate (which can form ICLs) is most likely not directly connected to the recombination/repair or gene correction mechanism. Previous reports on repair of psoralen-TFOs and chlorambucil-TFOs in human cell extracts have shown that such complexes may be processed by the cellular repair machinery, resulting in mutagenesis of the targeted DNA. However, the physical nature of PNA may explain the apparent minimal processing with the present PNAs (psp-PNA and chl-PNA). The 15mer PNA oligomers form triplexes with far higher binding affinity than regular oligonucleotides and are fully nuclease and protease resistant. Therefore, PNAs which can form covalent bonds to DNA may be close to “inert” to cellular DNA repair mechanisms, or may at least be very slowly processed by such mechanisms. Furthermore, as triplex conjugated chlorambucil are expected to form mono-adducts rather than cross links (as psoralen) with dsDNA, chlorambucil-PNA complexes may be more easily processed by the cellular repair machinery. This might explain why treatment with chlorambucil-PNA, compared to psoralen-PNA, results in a relatively smaller reduction of the gene correction frequency.

The apparent absence of psoralen-PNA processing may relate to the findings of Wang et al. describing the repair of psoralen-TFOs in HeLa cell extract. These authors reported that whereas a 10mer psoralen-conjugated TFO could be efficiently removed.
The observation that psoralen-PNA decreases the level of gene correction is somewhat in contrast to results from Faruqi et al. who reported that a psoralen-conjugated TFO could increase the levels of intra-molecular gene correction. It may be argued that differences in the experimental setup could explain the differences in the results (these authors used an episomal vector system with tandem repeat mutated genes). Because psoralen-conjugated TFOs on episomal vectors are encountered by helicases during replication, these enzymes may aid unwinding of triplexes, and stalled replication forks may stimulate recombinatory mechanisms. We have studied triplexes on non-replicating vectors, which do not normally encounter helicases. However, previous studies have shown that PNA-DNA complexes do not constitute good substrates for DNA helicases. Future experiments will determine if PNAs that can react covalently with DNA are as perseverant in a genomic context and on replicating episomes. If so, instead of exploiting TFP-psoralen conjugates in gene repair contexts, such PNAs may in principle be developed into gene specific anticancer-drugs, since replication blocks are not tolerated in mammalian cells and lead to apoptosis or inhibition of cell division. As cells will tolerate approximately 2,500 ICLs per genome, the tolerance for psoralen-PNAs or chlorambucil-PNAs may be significantly lower. In addition, ICL resistant cancer cells may not be resistant to treatment with PNAs that can form ICLs. Further studies are warranted to elucidate these aspects.

In contrast, the results using the camptothecin-PNA oligomer show that PNA conjugated to this topoisomerase I inhibitor significantly increased recombination frequency in human cell extract, and the effect could be augmented by ubiquitin supplementation of the extract. Previously, camptothecin-TFOs have been shown to trap topoisomerase I proximal to the triplex complex in cell nuclei and induce site specific, genomic cleavage. The present results for the first time demonstrate than camptothecin-PNA complexes may significantly increase gene correction rates. We suggest that the observed effect from camptothecin-PNA in the nuclear extract is connected to camptothecin interaction with topoisomerase I, which can lead to single strand breaks. This is in accordance with the generally accepted contention that DNA strand breaks can increase the frequency of genetic recombination events. The apparent absence of any such stimulatory effect in cells from the camptothecin-PNA conjugate could be caused by differences in repair processing of camptothecin-PNA in cells and in extracts. However, the stability of the camptothecin-PNA/vector complex during transfection and in the cell remains to be determined to ascertain that it is indeed present when the DNA vector reaches the nucleus. Also the complex or the PNA conjugate could be sequestered by protein binding or by other means before reaching the nucleus. Thus the effects and use of camptothecin conjugates in gene targeted repair merit further studies.

Figure 5. Correction rates in nuclear extract using the single stranded DNA donor #95. Correction rates using triplex forming PNA oligomers (black bars) are compared to the non-stimulated correction rate (white bar). *p < 0.05, **p < 0.01, ***p < 0.001. (n = 6).

Figure 6. Correction rates in nuclear extract using the double stranded DNA donor SxP34. Correction rates using triplex forming PNA oligomers (black bars) are compared to the non-stimulated correction rate (white bar). *p < 0.05, **p < 0.01, ***p < 0.001. (n = 6).
Materials and Methods

Plasmid vectors. The plasmid vector p25 was constructed by inserting a point mutated (A to T at position 79) EGFP gene into the pcDNA3.1-v5-His vector (Invitrogen). A target site for triplex binding was inserted upstream of the EGFP gene (Fig. 2). The plasmid vector p34 instead contains a non-mutated but truncated EGFP gene and a scrambled triplex binding target site.

Oligonucleotides were synthesized by MWG-biotech. The donor fragment #95 is a 53mer single stranded oligonucleotide, which shares full homology with bases 53–105 of the untranscribed strand of the EGFP gene (5'-AGC TGG ACG GCC ACG TAA ACG GCC ACA AGT TCA GCG TGT CCG GCG AGG CCG AG-3'). Oligonucleotides #39 (5'-AAA CAA ATA GGG GTT CCG CGC ACA TTT CC-3') and #106 (5'-CTT GTG CCC CAG GAT GTT GCC GTC CTC GTT G-3') were used to produce the double stranded PCR donor fragment SxP34 (Fig. 2). Both oligonucleotides were modified at the 5' end (phosphorothioate linkages at the four terminal residues) to resist exonuclease attack. T7 + primer: 5'-GCT TAT CGA AAT TAA TAC GAC TCA CTA TAG GG-3'; #96 + primer: 5'-TGG CCG TTT ACG TCG CCG TCC AGC-3'.

PNA synthesis. PNA synthesis was carried out by standard Boc strategy as previously reported in reference 17 and 18. Psoralen and camptothecin were linked to the PNA at the N-terminal via solid phase conjugation using carboxylic acid derivatives of the ligands. 10-Hydroxycamptothecin carboxylic acid was synthesized as described by Wang and Dervan.19 Chlorambucil was conjugated to the PNA in solution using HBTU coupling. Therefore γ-N-N-dimethyllysine (dMLys) was used instead of lysine for the PNA (PNA3348: H-eg1-(dMLys)-TTTTTTJTTJTJTTJTT-eg1-NH2): To 1.3 mg PNA3348 dissolved in 100 μL DMSO was added 2.3 mg HBTU in DMF (450 μL) and subsequently 2.4 mg chlorambucilbutanoic acid dissolved in 100 μL DMSO and 20 μL DIEA. The reaction was incubated at 20°C for 3 h and purified by HPLC.

The PNAs were equipped (on the solid support) with a spacer composed of ethyleneglycol linkers (eg1) between the PNA and the ligand. The PNA conjugates were purified by HPLC and characterized by MALDI-TOF mass spectrometry (see Table 1). The PNAs were lyophilized and stored at 4°C until use.

PCR fragments. The PCR donor fragment SxP34 was produced using the plasmid vector p34 as template and oligonucleotide primers #39 and #106. Following PCR amplification, the 1,650 bp fragment was purified on a Kleen spin column (Bio-Rad) and quantified.

Nuclear extract. The HeLa cell nuclear extract was obtained from Cil Biotech (Belgium). Purified ubiquitin was obtained from Boston Biotech.

Figure 7. Effect of ubiquitin on PNA3250 stimulation of gene correction in nuclear extract. *p < 0.05. (n = 6).

Figure 8. Measurement of gene correction in HeLa cells using FACS. EGFP fluorescence is measured on the X-axis and propidium staining (viability) is measured on the Y-axis. Cells in the R1 area are scored as positives for gene correction. (A) Mock transfected (p25) HeLa cells. (B) Cells transfected with p25/PNA3250 and SxP34 (Fig. 9). A total of 50,000 cells counted.
Cells. HeLa cells were obtained from American Type Culture Collection (CCL-2). DC-3F cells were a gift from Dr. Julie Gehl at Herlev Hospital, Copenhagen Denmark. Cells were grown in growth media (Dulbecco’s modified Eagle’s medium), 10% foetal calf serum and 1% penicillin/streptomycin in a humidified incubator at 37°C, 5% CO₂.

Gel mobility shift assay. The oligonucleotide primer T7+ was end labeled with γ-32P-ATP using T4 polynucleotide kinase. The labeled primer was used, without purification, in a PCR reaction including primer #96+ and p25 as template. The end-labeled PCR fragment (150 bp) contains the target site for triplex formation. In a total volume of 10 μl, a final concentration of 0.5 μM labeled PCR fragment was incubated with varying concentrations of PNA oligomers. Following a 1 h incubation in 10 mM Na₃HPO₄, pH 7.2 at 37°C, the samples were analyzed by electrophoresis overnight in a native 12% polyacrylamide gel with 1x TBE. The gel was vacuum-dried and analysed using autoradiography.

In vitro triplex formation. p25 (78 nM) was incubated with a six-fold excess (480 nM) of various PNA oligomers in 10 mM Na₂HPO₄, pH 7.2 for 1 h at 37°C. Following incubation, psoralen-PNA (PNA3084) triplexes were photo-activated by UV-irradiation (365 nm) for 1 h. Samples were placed on ice during UV-irradiation.

Nuclear extract gene correction assay. Reactions were performed in a total volume of 50 μl containing 1 μg plasmid vector p25 (± PNA oligomers), 1 μg of donor fragment (#95 or SxP34), 60 μg nuclear protein, 12 mM MgCl₂, 36 mM NaCl, 29 mM KCl, 4 mM HEPES (pH 7.2), 4% v/v glycerol, 0.1 mM DTT, 0.1 mM PMSF, 0.1 mM dNTP mix, 0.4 mM NTP mix (ATP 2.4 mM), 6 mM creatine phosphate and 1.25 μg creatine phosphokinase. Three μg (3.52 μM) of ubiquitin was added when stated. Following incubation at 37°C for 2 h the reactions were stopped by the addition of 250 μl termination buffer (20 mM HEPES (pH 7.9), 1% (v/v) SDS and 20 mM EDTA) and 2 μg tRNA. DNA was subsequently isolated using phenol/chloroform extraction, ethanol precipitation and dissolved in 20 μl H₂O. Chemically competent E. coli DH5-α cells (40 μl) were transformed using ~80 ng of purified DNA. Cells were grown in selection media overnight at 37°C in a shaker. Just prior to FACS analysis, 200 μl of the overnight culture was diluted into 1 ml of ice-cold PBS (1% FBS), followed by short vortexing. Samples were analysed using a FACScalibur (BD Biosciences) flow cytometer, in which the cells were initially analysed by forward and side scatter. The largest cluster of bacteria cells were selected, and subsequently analysed for green and red fluorescence emission, after appropriate fluorescence correction. Cells emitting higher levels of green fluorescence than mock-transfected cells were scored as positives for gene correction. Side scatter, forward scatter, and fluorescence

![Gene correction frequency (%)](image)

**Figure 9.** Correction rates in HeLa using the double stranded DNA donor SxP34. Correction rates using triplex forming PNA oligomers (black bars) are compared to the non-stimulated correction rate (white bar). *p < 0.05, **p < 0.01, ***p < 0.001 (n = 6).

| Table 1. PNA conjugates |
|------------------------|
| No | PNA | HPLC purity | Mass found (calc.) |
|---|---|---|---|
| 3051 | H-(Lys)₃-TTTTTJTJTJ-T-Lys-NH₂ | 90%* | 4448 (4448) |
| 3084 | Psol-egl1-(Lys)₃-TTTTTJTJTJ-T-Lys-NH₂ | 95%* | 4981 (4980) |
| 3250 | Camptothecin-egl1-(Lys)₃-TTTTTJTJTJ-T-Lys-NH₂ | 80%* | 5142 (5142) |
| 3349 | Chlorambucil-egl1-(DM-Lys)₃-TTTTTJTJTJ-T-DM-Lys-NH₂ | 75%* | 5041 (5040) |
| 3348 | H-egl1-(DM-Lys)₃-TTTTTJTJTJ-T-DM-Lys-NH₂ | 90%* | 4753 (4755) |

*One major peak and a few minor impurities; +One major peak and a minor impurity; *One major impurity (which was not detected by MALDI mass spectrometry, and which may have formed during purification) was present.
data were collected using logarithmic amplification. One hundred thousand cells were analysed for each reaction sample.

**Cellular gene correction assay.** According to the transcription protocol from Invitrogen, 5 × 10^4 cells were co-transfected with 0.1 μg plasmid vector (p25 ± PNA oligomers) and 0.1 μg donor fragment (#95 or SixP34) using Lipofectamine 2000 (Invitrogen). After incubation for 96 h, cells were harvested, re-suspended in 1 ml of ice-cold PBS (1% FBS) and passed through a cell strainer. Just prior to FACs analysis, 2 μl (1 mg/ml) propidium iodide (PI) was added. Cells were then analysed using a FACScalibur (BD Biosciences) flow cytometer, in which the cells were initially analysed by appropriate forward and side scatter. The population of single cells was selected for further analysis. This population of cells was analysed for green and red fluorescence emission, after appropriate fluorescence correction, and cells that were PI negative, and emitting higher levels of green fluorescence than mock transfected cells, were scored as positives for gene correction. Side scatter, forward scatter and fluorescence data were collected using logarithmic amplification. Fifty-thousand cells were analysed for each reaction sample.

**Data analysis.** All statistical analysis (unpaired student’s t test) and creation of graphs were performed using Graph Pad. Error bars on graphs indicate SEM.

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Note

Supplemental materials can be found at: www.landesbioscience.com/journals/artificialdna/article/15553

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