Nanoparticle for delivery of antisense γ PNA oligomers targeting CCR5

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The development of a new class of peptide nucleic acids (PNAs), i.e., gamma PNAs (γ PNAs), creates the need for a general and effective method for its delivery into cells for regulating gene expression in mammalian cells. Here we report the antisense activity of a recently developed hydrophilic and biocompatible diethylene glycol (miniPEG)-based gamma peptide nucleic acid called ^{MP} γ PNAs via its delivery by poly(lactide-co-glycolide) (PLGA)-based nanoparticle system. We show that ^{MP} γ PNA oligomers designed to bind to the selective region of chemokine receptor 5 (CCR5) transcript, induce potent and sequence-specific antisense effects as compared with regular PNA oligomers. In addition, PLGA nanoparticle delivery of ^{MP} γ PNAs is not toxic to the cells. The findings reported in this study provide a combination of γ PNA technology and PLGA-based nanoparticle delivery method for regulating gene expression in live cells via the antisense mechanism.

Introduction

Antisense strategies, where synthetic oligonucleotides are used to target RNA selectively, provide new avenues for controlling gene expression and regulation.¹ However, there are major problems with conventional synthetic DNA- and RNA-based oligonucleotide approaches including enzymatic degradation,² non-specific binding,³ and poor cellular uptake.⁴ Several promising classes of nucleic acid analogs, including morpholinos,⁵ phosphorothioates,⁶ and locked nucleic acids (LNAs)⁷ have been reported to improve the enzymatic stability of oligonucleotides. Similarly, one such emerging class is peptide nucleic acids (PNAs), which has gained attention as an effective antisense agent over the last two decades.⁸ Structurally, PNA is a nucleic acid (DNA or RNA) analog in which the sugar phosphodiester backbone is replaced with homomorphous achiral N-(2 aminoethyl) glycine units. In addition, the most salient features of PNA include: (1) its propensity to hybridize with DNA and RNA with higher affinity and selectivity based on the rules of Watson-Crick base pairing; and (2) its charge neutral structure makes it resistant to degradation by proteases as well as nucleases. In addition to effective antisense, PNA has also been employed as a recognition molecule⁹ and molecular assembly tool in drug discovery and nanotechnology.¹⁰ However, in spite of these attractive features and numerous applications, a few technical challenges still circumscribe the development of PNA as a therapeutic molecule for clinical application. These challenges include: (1) low solubility,

and (2) difficulty in intracellular delivery. In attempts to resolve these limitations, strides have been made by incorporation of numerous cationic residues,¹¹ inclusion of polar groups in the backbone¹² and nucleobases,¹³ and conjugation of high molecular weight polyethylene glycol (PEG) to one of the termini.¹⁴ Though these chemical modifications have led to improvements in solubility and cellular uptake properties, it is often achieved at the expense of binding affinity and sequence specificity.

These limitations can be addressed by inducing chirality at the gamma position of regular PNA: one class of chiral PNA is known as gamma PNA (γ PNA). Biophysical characterization and NMR structural studies have shown that installation of a chiral center at the gamma position pre-organizes the PNA oligomer and also increases the binding affinity to the cDNA or RNA sequences.^{15–20}

Recently, by performing a series of thermodynamic studies, we have revealed that inclusion of diethylene glycol at the gamma position (also known as miniPEG-based γ PNA) increases the solubility properties of PNA and enhances its binding with cDNA and RNA strands.¹⁷ In addition, we have also shown that miniPEG-based gamma PNA (^{MP} γ PNA) has the potential to invade duplex DNA in a sequence-unrestricted manner.²¹ However, more effective intracellular delivery methods are required for broader impact of new generations of gamma PNA in gene therapy-based applications.

Recently, we have developed new methods for delivery of PNAs for gene silencing and gene editing. McNeer et al. demonstrated the use of poly(lactide-co-glycolide) (PLGA) nanoparticles for

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Figure 1. (A) Human CCR5 genomic DNA and transcribed mRNA transcript (GenBank accession number AF011539). (B) Chemical structure of unmodified PNA, ^{MP}_γPNA, ^{MP}_γGPNA units. (C) Designed oligomer sequence of unmodified and gamma-modified PNA oligomers to target mRNA transcript. Bold letters indicate ^{MP}_γPNA units, Bold underline indicates ^{MP}_γGPNA units, and bold underlined and italics indicates the mismatch sites. All the PNAs positions are from 677 to 693 as shown above.

delivery of PNA molecules for site-specific gene editing both in vitro and in vivo in relevant human cell types.^{22,23} Cheng et al. demonstrated the ability of PNA-loaded PLGA nanoparticles to modulate gene expression in vivo.²⁴

In this report, we use PLGA nanoparticles to deliver ^{MP}_γPNA. As a proof of concept, we employed CCR5 mRNA knockdown as a functional endpoint. CCR5 is a membrane protein receptor, which is required by R5-tropic HIV-1 virus to gain entry into CD4⁺ T-cells.²⁵ When the CCR5 receptor is unavailable, HIV virus cannot engage with CD4⁺ T-cells to infect the cell. The delta 32 mutation in CCR5 is a 32-base pair deletion in the genetic code that has previously been shown to confer resistance to HIV infection, showing that genome-level mutations in CCR5 can confer HIV resistance.²⁵ Our group has already shown that transfection of human cells with triplex-forming PNAs targeted to the CCR5 gene, plus donor DNAs designed to introduce stop codons to block CCR5 production, produced 2.46% targeted gene modification.²⁶ Here we use a different approach—gene knockdown rather than gene editing to reduce expression of CCR5. We employ RT-PCR analysis to show that our antisense gamma PNA, delivered into cells by PLGA nanoparticles, can decrease CCR5 mRNA levels.

Results

Target selection and PNA design. The PNA and ^γPNA sequences were designed to bind the mRNA transcript formed

during transcription from the template strand of human CCR5 genomic DNA. To compare the binding affinity and antisense efficiency of PNA vs. ^γPNA, three oligomers were designed to bind the complementary regions of the mRNA transcript (Fig. 1). PNA1 consisted of regular PNA units, ^{MP}_γPNA2 consisted of all miniPEG-based gamma PNA, and ^{MP}_γGPNA3 consisted of miniPEG gamma monomer units with inserted guanidinium ^γPNA units. In order to determine the selectivity of ^γPNA oligomers, mismatched ^γPNA (MM-^{MP}_γPNA4) was also synthesized. Inclusion of a lysine residue at the C-terminus as well as the N-terminus was necessary because attempts to synthesize regular PNA oligomers without the lysine residue resulted in sticky and poorly water-soluble materials that were difficult to purify and characterize. For cellular uptake studies, carboxy tetramethylrhodamine (TAMRA) derivatives were made by coupling the dye at the N-terminus of the ^{MP}_γGPNA3. All PNA oligomers were synthesized on a solid-support according to the published procedures of Christensen and coworkers based upon Boc-based synthetic protocols.²⁷ No precaution was necessary for coupling of ^γPNA monomers on-resin in terms of side reactions. Upon completion of the synthesis, the oligomers were cleaved from the resin and precipitated with ethyl ether.

After air-drying, the crude pellets were dissolved in water/acetonitrile mixture (80/20), purified by reverse-phase HPLC, and characterized by MALDI-TOF mass spectrometry.

Thermal stability analysis. UV-melting experiments were performed to determine the effect of preorganization and cationic properties of ^γPNA upon hybridization to complementary RNA nucleobases through Watson-Crick base pairing as compared with the regular PNA units. Samples containing stoichiometric amounts of PNA and RNA (5 μM strand concentration each) were prepared in 10 mM NaPi buffer and annealed, and their UV absorption at 260 nm was recorded as a function of temperature for both the heating and the cooling runs. Figure 2 shows the heating profiles of the various PNA-RNA hybrid duplexes, compared with the corresponding ^γPNA-RNA duplex. A significant stabilization was observed for the ^{MP}_γPNA2-RNA and ^{MP}_γGPNA3-RNA duplexes, with T_m > 95°C as compared with ~84°C for PNA1-RNA corresponding to a net gain in T_m > 20°C. Our results clearly indicate that incorporation of a miniPEG side chain as well as cationic moieties (guanidinium) significantly increased the binding affinity of ^{MP}_γPNA2 and ^{MP}_γGPNA3 as compared with the regular PNA oligomer.

Nanoparticle synthesis and nanoparticle release profile data. To enhance the cellular delivery, PNA and ^γPNA oligomers were incorporated into PLGA nanoparticles by a double-emulsion solvent evaporation technique.²² All nanoparticle preparations were of similar size, around ~150 nm, with uniform spherical morphologies, as determined by SEM (Fig. 3).

The kinetics of PNA/ γ PNA nucleic acid release from nanoparticles were determined by incubating 4–6 mg particles in 300 μ l PBS in 37°C shaker, spinning down and periodically removing supernatant to measure absorbance at 260 nm. More PNA1 and $^{MP}\gamma$ PNA2 were released as compared with $^{MP}\gamma$ GPNA3 for the 48-h time period (Fig. 4).

Cellular uptake studies. Intracellular delivery of PNA is difficult due to its charge neutral backbone. However in two separate previous studies, we have shown that PNA oligomers 15–17 mer in length and containing guanidinium groups at the gamma position, i.e., to read guanidine-based PNA (GPNA), are readily taken up by mammalian cells.^{28–30} Unfortunately, GPNA requires long incubation (48–72 h) at high concentration for biological activity. To assess cellular uptake, nanoparticles loaded with TAMRA-conjugated $^{MP}\gamma$ GPNA3 and naked $^{MP}\gamma$ GPNA3 (Fig. 1C) were incubated with HeLa cell lines for 24 h, stained with DAPI, and imaged by confocal microscopy. We noticed that naked $^{MP}\gamma$ GPNA3 also traversed the cell membrane, which was consistent with our previous findings.³⁰ However, we noticed considerably more fluorescence due to TAMRA in the nanoparticle-based delivery method (Fig. 5). No auto fluorescence was observed in the un-transfected cells in the TAMRA channel (data not shown).

Quantification of CCR5 mRNA and protein in THP1 cell lines. Next, we investigated the antisense activity of PNA and γ PNA encapsulated in nanoparticles on CCR5 mRNA levels by quantitative RT-PCR analysis. For all experiments, blank nanoparticles were employed as controls. Because human peripheral blood white cells are the principal cells that express CCR5, we used quantitative RT-PCR to examine CCR5 expression in the THP1 myelomonocytic cell line. The THP-1 monocyte cell line, derived from an individual with monocytic leukemia, is readily converted to a macrophage-type cell in the presence of phorbol myristate acetate (PMA).^{31,32} PMA (50 ng/ml) promotes differentiation of THP-1 cells into a macrophage-like state, thereby yielding high levels of CCR5 cell surface expression after 48 h treatment. THP1 cell lines were treated with nanoparticles at a dose of 3 mg nanoparticles per million cells for either 10 or 24 h, which contained approximately a 4 μ M concentration of PNA/ γ PNA oligomers. RNA was extracted and quantified by RT-PCR including GAPDH as a control. We found that after 24 h treatment, a ~40% CCR5 mRNA reduction was observed for $^{MP}\gamma$ PNA2-loaded and $^{MP}\gamma$ GPNA3-loaded nanoparticles (Fig. 6). [Note that the RT-PCR primers amplify a region (512–668) in CCR5 that is upstream of the PNA binding site (677–693) and that there is no overlap with this site.] However, we did not notice any decrease in RNA level by nanoparticles containing regular PNA1. These results suggest that the gamma-modified PNA oligomers show more pronounced antisense activity, presumably related to the preorganization properties and higher binding affinity.

In order to confirm the selectivity, mismatched oligomer (MM- $^{MP}\gamma$ PNA4) was synthesized followed by its encapsulation in PLGA nanoparticle. Further RT-PCR analysis was performed with mismatched oligomers enclosed in PLGA nanoparticles. We did not notice any considerable decrease in RNA level with mismatched oligomer (Fig. 7).

Figure 2. UV-melting profiles of PNA-RNA hybrid duplexes at 5 μ M strand concentration each in 10 mM sodium phosphate buffer at pH 7.4. Both the heating and cooling runs were performed; they both had nearly identical profiles (only the heating runs are shown).

Similarly, we also performed a dose-response experiment where THP1 cells were treated with different doses of nanoparticles and incubated for 24 h. RT-PCR analyses were performed showing decreased mRNA levels as the doses increased (Fig. 8).

These results were further corroborated with a subsequent decrease in CCR5 protein levels after 24 h treatment as shown in western blot analysis (Fig. 9), consistent with an anti-sense mode of action via inhibition of translation.

Cell survival data. Cell survival was determined for cells harvested from each THP1 cell pool after intervals of 10 and 25 h indicating that PLGA nanoparticles containing $^{MP}\gamma$ PNAs do not exert any toxic effects (Fig. 10).

Discussion

Two major challenges in the field of PNA-based therapeutics are cellular delivery and solubility of PNA oligomers. In order to resolve issues regarding cellular delivery, several promising chemical, mechanical and/or electrical transduction means are generally employed to transport these PNA oligomers. However, despite their promising results, these methods cannot be used for many in vivo experiments or therapeutic and diagnostic applications. In addition, the use of exogenous transduction reagents often leads to off-target and cytotoxic effects. Similarly, classes of cationic PNAs have been reported that can aid cellular uptake, but at the expense of its binding affinity, which further leads to toxicity. In order to overcome the solubility issues, gamma position switch in the regular PNA has been explored. In previous reports, we have shown that a randomly folded PNA can be pre-organized into a right-handed helix by installing a miniPEG side-chain at the γ -backbone: this modification also increases its solubility by several fold.^{17,21} These enhancements in water solubility and biocompatibility will facilitate the handling and

Figure 3. Nanoparticles show uniform size and morphology. Scanning electron microscope (SEM) images of PNA and γ PNA nanoparticle batches. Average particle diameter and SD given under each batch.

Figure 4. PNA and γ PNA nanoparticle release profile data of nucleic acid after indicated time points in a graph and incubation at 37°C.

processing of PNA, lessening the concerns for nonspecific binding and cytotoxic effects. Still, effective formulations to increase the cellular uptake of gamma PNA oligomer are essential to test its efficacy at the clinical level.

Our group has also shown that inserting a few guanidine groups in the oligomers can increase the cell membrane permeability of gamma PNA.²⁸⁻³⁰ This strategy has been shown to be promising but requires synthesis of a different class of gamma PNA monomers. In addition, based on molecular targets, to read guanidine-based PNA oligomers require long hours of incubation for exerting their antisense effect. As an alternate, our group has also previously shown that PLGA nanoparticles provide an effective and non-toxic delivery mechanism for PNA molecules.

In this report we have combined two approaches, using PLGA-based nanoparticles as a carrier and miniPEG-based gamma PNA as a new strategy to control gene expression. PLGA has long been approved for use in drug delivery in humans, and the use of PLGA NPs for delivery of DNA plasmids³³ and siRNA³⁴ has already been demonstrated. Moreover, PLGA nanoparticles are relatively simple to synthesize. Recent studies have shown that nanoparticles loaded with conventional PNA can be used to knock down miRNA-155 levels in tumor cells both in vitro and in vivo.³⁵ Here, moving a step forward in the applicability of gamma PNA oligomers, we have demonstrated that miniPEG

gamma PNA oligomers have much stronger binding affinity as compared with the regular PNA oligomers and that they block the mRNA activity based on steric blockage mechanism.

All of our PNA molecules were efficiently loaded into PLGA nanoparticles. SEM characterization showed that PLGA NPs loaded with gamma PNA had an average size of 150–200 nm. When incubated in buffered saline, PNA was released from the nanoparticles; miniPEG side chains at the gamma position have no dramatic effect on the PNA release from the PLGA polymer as compared with regular PNA oligomer. However we noticed that miniPEG PNA containing inserted guanidinium groups was released in lower amounts. One possible explanation for this observation is that the positively charged guanidinium heads interact with PLGA polymer, causing release at a slower rate as compared with fully modified hydrophilic miniPEG-containing PNA oligomers.

To assess cellular permeability, we performed cellular uptake assays of naked vs. nanoparticle-loaded gamma PNA. Previously it has been shown that guanidinium groups increase cellular uptake of $MP\gamma GPNA$ over the course of a 24 to 48 h treatment. Here, it is worth noting that HeLa cell lines treated with $MP\gamma GPNA3$ encapsulated PLGA-based nanoparticle leads to more cellular uptake of PNA as compared with treatment with naked $MP\gamma GPNA$. Further, PLGA-based gamma PNA nanoparticles are not toxic to cells even after 10 and 24 h treatment.

As proof of concept for downregulation of CCR5 expression by gamma PNA, we first incubated nanoparticles with THP1 cells. THP1 is an acute monocytic leukemia cell line that expresses the CCR5 gene. We observed a ~40% reduction in CCR5 level. Mismatch and western blot studies have clearly demonstrated that designed antisense gamma PNA oligomers bind through antisense mechanisms and bring down the CCR5 protein level sequence selectively.

In previous studies, siRNA-based strategies have been employed to silence the gene expression of CCR5.³⁶ However, studies demonstrated in this report are based upon a different mechanism where CCR5 gene knockdown was elicited due to PNA steric hindrance between mRNA and ribosomal units; and unlike siRNA technology, here only one strand of PNA has been used to elicit their effect.

In the antisense field, the first known basic mechanism revealed by oligodeoxynucleotides is considered to be a ribonuclease H (RNase H)-mediated cleavage of the RNA strand in the oligonucleotide-RNA heteroduplex of the translation machinery.^{37,38} Nucleic acid analogs such as phosphorothioates activate RNase H and exhibit their antisense effect.^{39–41} However, nonspecificity is still a major issue that needs to be resolved for RNase-based antisense technology.⁴² It has been well documented that PNA/RNA duplexes cannot act as substrates for RNase H and antisense effect

Figure 5. Fluorescent confocal live-cell images of HeLa cells following 24 h incubation with nanoparticles containing $MP\gamma GPNA3$ and naked $MP\gamma GPNA3$.

Figure 6. CCR5 mRNA expression in THP1 cells after treatment with PLGA nanoparticles containing PNA as indicated in X-axis. CCR5 expression relative to average blank control (all normalized to GAPDH, $n = 3$, $p < 0.05$).

is based on the steric blocking of translational machinery.^{43–45} In addition to mixed purine/pyrimidine sequence-forming PNA duplexes, homopyrimidine PNAs forming (PNA)₂/RNA triplex structures are also not substrates for RNase H degradation.⁴⁴ It has been shown that targeting regular PNA to the region spanning the AUG initiation codon brings effective inhibition of translation initiation in vitro successfully.^{46–49} In addition, several in vivo studies have also demonstrated that regular PNA targeting the start codons of mRNA leads to promising antisense effect based on translation inhibition.^{50–52} The fact that the start codon site of the mRNA, in contrast to other sites, is sensitive to antisense PNA inhibition presumably because it requires lower duplex (PNA/RNA) stability to prevent ribosome assembly rather than to prevent ribosome elongation.

In this manuscript, we have shown the gamma PNA designed to other target sites can also bring down the mRNA level based

on nanoparticle-based in vitro experiments. The most reasonable explanation for this argument: in the past we have shown that gamma PNA possesses the considerably high binding affinity to complementary sequences as compared with regular PNA oligomers because of its preorganization properties. Moreover, gamma PNA designed for our study consists of preorganization as well as high cationic behavior because of lysine and guanidium residues which probably contribute to high binding toward complementary targeted sequences.

We successfully demonstrated that nanoparticle-based delivery method of gamma PNA can decrease the CCR5 expression by targeting PNA in the middle of mRNA transcript. Nonetheless, more effective strategy can be devised to target the transcription and translational start sites of mRNA by employing gamma PNA oligomers. Results presented in this report can serve as a promising platform based on the nanoparticle/gamma PNA technology to control the gene expression and regulation.

In conclusion, our data demonstrate that miniPEG-based gamma PNA oligomer-targeting sequences can be efficiently delivered by nanoparticles. This approach can be used to effectively reduce the levels of CCR5 mRNA expression via antisense mechanism. Improvements in accessibility to molecular targets and increased specificity could further be increased by coating nanoparticles with specific peptides, antibody and carbohydrate units. In summary, this work provides a novel platform for gamma PNA delivery that can be developed for therapeutic purposes.

Materials and Methods

PNA monomer synthesis. Regular Boc-protected PNA monomers were purchased from ASM Research Chemicals. MiniPEG-containing γ PNA ($^{MP}\gamma$ PNA) monomers were synthesized using Boc-protected *L*-serine as a starting material, as previously reported by Sahu and coworkers. γ GPNA monomers were synthesized using Boc-protected *L*-lysine as a starting material, as reported by Sahu and coworkers.¹⁷

PNA synthesis. All oligomers were synthesized on solid support using standard Boc chemistry procedures. The oligomers were cleaved from the resin using *m*-cresol:thioanisole:TFMSA:TFA (1:1:2:6) cocktail solution. The resulting mixtures were precipitated with ether (3 $\times$), purified and characterized by RP-HPLC and MALDI-TOF, respectively. All PNA stock solutions were prepared using nanopure water and the concentrations were determined at 90°C on a Cary 3 Bio spectrophotometer using the following extinction coefficients: 13,700 M⁻¹cm⁻¹ (A), 6,600 M⁻¹cm⁻¹ (C), 11,700 M⁻¹cm⁻¹ (G), and 8,600 M⁻¹cm⁻¹ (T). 5-Carboxytetramethylrhodamine (TAMRA) purchased from VWR was exclusively conjugated to the N-terminus of PNAs. TAMRA-conjugated PNA concentration was measured by using extinction coefficients of rhodamine: 91,000 M⁻¹cm⁻¹ at 560 nm.

PNA nanoparticle (PNA-NP) synthesis. PLGA nanoparticles containing all PNAs listed in Figure 1 were formulated using the double-emulsion solvent evaporation method as previously described.⁵³ PNAs were dissolved in 60.8 μ l DNase/RNase-free water, and all nanoparticle batches had 2 nmole/mg of PNA or

Figure 7. CCR5 mRNA expression in THP1 cells after treatment with $^{MP}\gamma$ GPNA3 and MM- $^{MP}\gamma$ PNA4 containing PLGA nanoparticles. CCR5 expression relative to average blank control (all normalized to GAPDH, n = 3, *p < 0.05).

Figure 8. Dose-dependent effect in CCR5 mRNA expression level in THP1 cell lines after treatment with different doses of $^{MP}\gamma$ GPNA3-containing PLGA nanoparticles. CCR5 expression relative to average blank control (all normalized to GAPDH, n = 3, *p < 0.05).

Figure 9. Western blot showing the corresponding changes of CCR5 protein levels in THP1 cells after treatment with blank, PNA1 and $^{MP}\gamma$ GPNA3-containing nanoparticles for 24 h.

γ PNA. The encapsulant in water was added dropwise to a polymer solution of 80 mg 50:50 ester-terminated PLGA dissolved in 800 μ l dichloromethane (DCM), then ultrasonicated (3 $\times$ 10 sec) to form the first emulsion. This emulsion was added slowly dropwise to 1.6 ml of 5% aqueous polyvinyl alcohol then ultrasonicated (3 $\times$ 10 sec) to form the second emulsion. Subsequently this mixture was poured into 20 ml of 0.3% aqueous polyvinyl alcohol and stirred at room temperature for 3 h. Nanoparticles were then washed with 20 ml water three times and collected each time by centrifugation at 12,000 rpm for 10 min at 4°C. Then

nanoparticles were resuspended in water, frozen at -80°C , and lyophilized. Particles were stored at -20°C following lyophilization.

Release of PNAs from nanoparticles. Release of PNAs was analyzed by incubating 4–6 mg particles in 600 μl PBS in a 37°C shaker, spinning down, and removing supernatant to measure absorbance 260 nm at indicated time points. Absorbances at 260 nm were then measured with a Nanodrop 8000 (Thermo Fisher Scientific).

Differentiation of THP1 cell lines into macrophages after treatment with PMA. THP-1 cells were induced to differentiate into adherent macrophage-like cells by treatment with PMA. Cells were plated at 1×10^6 cells/well in 12-well plates and treated with 50 ng/ml PMA for 48 h at 37°C and washed thoroughly.

PNA-NP imaging and determination of particle size. A sample of particles from each batch was also analyzed using scanning electron microscopy (SEM). Samples were coated with 25-nm thick palladium using a sputter coater. Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD), with > 300 particles analyzed per batch to determine size distribution. Briefly, brightness, contrast, and threshold were adjusted to enhance particle outlines, and then ImageJ's "Analyze Particles" function was used to calculate the area of each particle.

Cell culture. HeLa cells were grown in DMEM with 10% fetal bovine serum (Gibco). Cell counts were performed using a Cellometer Auto T4 from Nexcelom Bioscience. THP1 cells were grown in RPMI with 10% fetal bovine serum.

Real time reverse-transcription quantitative PCR. At each timepoint, cells were harvested, pelleted, and stored frozen in RNA stabilization reagent (Qiagen), until ready for RNA extraction. RNA was extracted from the cell pellets using the RNeasy Mini Plus kit from Qiagen, as per the manufacturer's protocol. The Invitrogen SuperScript III kit was used to generate cDNA from the RNA, as per the manufacturer's protocol, using 500 ng of RNA per reaction. PCR reactions contained cDNA, 20% Betaine, 0.2 mM dNTPS, Advantage 2 Polymerase Mix, 0.2 μM of each primer, 2% Platinum Taq, and Brilliant SYBR Green and ROX reference dye from Stratagene, on the Mx3000p real-time cycler. Cycler conditions were 94°C for 2 min, 40 cycles of 94°C 30 s/ 50°C 30 s/ 72°C 1 min, then 95°C 1 min. Amounts of CCR5 relative to GAPDH were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method and then normalized to untreated controls to calculate percent expression.

Primers used were as follows:

CCR5-up: 5'-CAAAAGGAGG TCTTCATTAC ACC-3' (position, 512 to 535).

CCR5-dn: 5'-AGAGTTTTTA GGATTCCTGA GTA-3' (position, 645 to 668).

GAPDH-sense: 5'-TGATGACATC AAGAAGGTGG TGAAG-3'.

Figure 10. Cell survival graph data for THP1 cell lines after 10 h and 25 h treatment with nanoparticles containing PNA/γPNA oligomers.

GAPDH-antisense: 5'-TCCTTGGAGG CCATGTGGGC CAT-3'.

Confocal imaging. HeLa cells were treated with 2 mg of nanoparticles containing $\text{MP}\gamma\text{GPNA3a-Tam}$ for 24 h. After 24 h, DAPI staining was performed followed by live-cell imaging. A Leica TCS SP5 Spectral Confocal Microscope was used to image slides, using the z-stack function to image slices near the middle of the cells.

Western blot data. THP1 cells were incubated with blank, PNA1 and gamma PNA nanoparticles and incubated for 24 h. After 24 h, cell pellets were collected and immediately placed on ice. After rinsing with phosphate-buffered solution (PBS) cells were scraped, collected, and used for protein extraction with AZ lysis buffer. 80 μg total protein was loaded and size fractionated via SDS/PAGE and transferred to a nitrocellulose membrane. Antibodies were as follows: rabbit monoclonal anti-CCR5-antibody (e164) (Abcam Laboratories; Cat: ab32048); mouse monoclonal anti- α -tubulin (B-5-1-2; Sigma-Aldrich); anti-mouse IgG (31432; Thermo Fisher Scientific).

Statistical analyses. Data were analyzed and plotted in Microsoft Excel 2010. Data are represented as means $\pm$ the standard deviation. Experiments were performed in triplicate ($n = 3$) unless otherwise noted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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