Polypurine Hairpins Directed against the Template Strand of DNA Knock Down the Expression of Mammalian Genes*

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We analyzed whether polypurine hairpins (PPRHs) had the ability to knock down gene expression. These hairpins are formed by two antiparallel purine domains linked by a loop that allows the formation of Hoogsteen bonds between both domains and Watson-Crick bonds with the target polypyrimidine sequence, forming triplex structures. To set up the experimental conditions, the human dhfr gene was used as a model. The PPRHs were designed toward the template strand of DNA. The transfection of the human breast cancer cell line SKBR3 with these template hairpins against the dhfr gene produced higher than 90% of cell mortality. Template PPRHs produced a decrease in DHFR mRNA, protein, and its corresponding enzymatic activity. In addition, the activity of DHFR PPRHs was tested against breast cancer cells resistant to methotrexate, observing high cell mortality. Given the difficulty in finding long polypyrimidine stretches, we studied how to compensate for the presence of purine interruptions in the polypyrimidine target sequence. The stability of PPRH was measured, resulting in surprisingly long half-life of about 5 days. Finally, to test the generality of usage, template PPRHs were employed against two important genes involved in cell proliferation, telomerase and survivin, producing 80 and 95% of cell death, respectively. Taken together our results show the ability of antiparallel purine hairpins to bind the template strand of double strand DNA and to decrease gene transcription. Thus, PPRHs can be considered as a new type of molecules to modulate gene expression.

Repression of gene expression serves as a powerful therapeutic tool to inhibit the synthesis of proteins involved in pathological processes. Among the different molecules that can repress gene expression, triplex forming oligonucleotides (TFOs) have shown promising results in inhibiting gene transcription (1–3). Triplex DNA is formed when a third DNA strand binds into the major groove of a double helix via Hoogsteen hydrogen bonding. The ability of nucleic acids to form triple helices was discovered in 1957 by Felsenfeld and Rich (4) and since then there have been numerous improvements in the field.

TFOs bind in a sequence-specific manner, but they present stability and binding affinity issues. To address these aspects, circular oligonucleotides (ODN) (5), hairpins, and more recently TFOs with locked nucleic acids (6, 7) were developed. Hairpins consist of two DNA strands linked by a loop (8–12), and can be either parallels or antiparallels. Antiparallel hairpins are more promising in the biomedical field, because triplex forms regardless of pH, whereas in most cases, parallel hairpins require an acidic pH (13–16). According to the rules of Hoogsteen bonds, antiparallel hairpins bind to polypurimidine stretches in the DNA target sequence, whereas parallel hairpins bind to polypurimidine stretches.

The intramolecular linkage of the hairpin is formed by reverse Hoogsteen bonds, and the union with the target sequence is mediated by Watson-Crick, d(G·G·C) and d(A·A·T). Accordingly, PPRHs (polypurine reverse Hoogsteen) are formed by two antiparallel homopurine domains linked by a five-thymidine loop (11, 17). One of the homopurine strands binds with antiparallel orientation (by Watson-Crick) to the polypurimidine target sequence forming a triplex. Although it might be thought as difficult to find polypurimidine stretches, triplex-forming oligonucleotide target sequences are more common in the human genome than predicted by random models, and they can be found mainly in regulatory regions, opening interesting possibilities for the use of triplexes in the control of gene expression (18, 19).

We previously reported the ability of PPRHs to effectively bind dsDNA (17) displacing the fourth strand away from the newly formed triplex. Here we describe the design and use of template PPRHs that bind to dsDNA as a new tool to decrease gene expression. This approach is different to that exerted by TFOs interfering with gene transcription (20–22). As a model, we used as targets polypurimidine sequences in the third intron of the dihydrofolate reductase (dhfr) gene, whose product is involved in the de novo synthesis of purines and TMP. We also evaluated the therapeutic ability of PPRHs targeting the telomerase and survivin genes. The cytotoxic activity of PPRHs when targeted at these essential genes was tested using human breast cancer cells. We also targeted the dhfr gene with PPRHs in cells resistant to methotrexate chemotherapy.

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4 The abbreviations used are: TFO, triplex forming oligonucleotide; PPRHs, polypurine reverse Hoogsteen hairpins; dhfr, dihydrofolate reductase; tetr, telomerase; birc5, survivin; apt, adenine phosphoribosyltransferase; aODN, antisense oligodeoxynucleotide; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N,N-trimethylammonium methysulfate; dsDNA, double-stranded DNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcriptase; siRNA, small interfering RNA; nt, nucleotide(s).
**PPRHs, A New Tool to Knock Down Gene Expression**

The success of the latter might open a new way to fight against cancer.

**EXPERIMENTAL PROCEDURES**

**PPRHs Design and Usage**

The PPRHs used in this study were made up of two antiparallel polypurine domains, bound by intramolecular reverse Hoogsteen bonds, and linked by a pentathymidine loop. The Triplex-Forming Oligonucleotide Target Sequence Search software, (Anderson Cancer Center; www.spi.mdanderson.org/tfo), was used to find the polypyrimidine stretches in the target sequence. BLAST analyses were performed to check for the specificity of all these sequences. Non-modified oligodeoxynucleotides were synthesized by Sigma Genosys (0.05 μmol scale). Purity was checked by gel electrophoresis. All concentrations were expressed in strand molarity. PPRHs were dis- solved in sterile RNase-free TE buffer (1 mM EDTA and 10 mM Tris, pH 8.0) and stored at −20 °C until use.

**Binding Analysis**

*Preparation of Polypurine/Polypyrimidine Duplexes—* The duplexes to be targeted by the hairpins were formed by mixing 25 μg of each single-stranded polypurine and polypyrimidine oligodeoxynucleotides in 150 mM NaCl. After incubation at 90 °C for 5 min, solutions were allowed to cool down slowly to room temperature. Duplexes were purified in a non-denaturing 20% polyacrylamide gel and quantified by their absorbance at 260 nm at 25 °C.

*Oligodeoxynucleotide Labeling—* One hundred ng of single or double-stranded oligodeoxynucleotides was 5’-end labeled with [γ-32P]ATP by T4 polynucleotide kinase (New England Biolabs) in a 10-μl reaction mixture, according to the manufacturer’s protocol. After incubation at 37 °C for 1 h, 90 μl of TE buffer (1 mM EDTA and 10 mM Tris, pH 8.0) was added to the reaction mixture, which was subsequently filtered through a Sephadex G-50 spin column (GE Healthcare) to eliminate the excess [γ-32P]ATP.

*Electrophoretic Mobility Shift Assay—* Triplex formation was analyzed by incubating radiolabeled single or double-stranded DNA targets (2 nM strand concentration; 20,000 cpm) in the presence or absence of unlabeled PPRHs (250 nM strand concentration) in a buffer containing 10 mM MgCl₂, 100 mM NaCl and 50 mM HEPES, pH 7.2. Binding reactions (20 μl) were incubated 45 min at 37 °C before the electrophoresis. Electrophoresis was performed on a non-denaturing 12% polyacrylamide gel containing 10 mM MgCl₂, 5% glycerol, and 50 mM HEPES, pH 7.2. Gels were run for 3–4 h at 190 V at 4 °C, dried, and analyzed on a Storm 840 PhosphorImager (GE Healthcare). Quantification was performed using the ImageQuant 5.2 software (GE Healthcare).

**Cell Culture**

SKBR3 breast cancer cells were grown in Ham’s F-12 medium containing 7% fetal bovine serum (Invitrogen), and incubated at 37 °C in a humidified 5% CO₂ atmosphere. MCF7-R are breast cancer cells resistant to 10⁻⁷ M methotrexate, generated in our laboratory, bearing amplification of the dhfr locus. This cell line was grown in Ham’s F-12 medium lacking the final products of DHFR activity: glycine, hypoxanthine, and thymidine (−GHT medium (23)) containing 7% of dialyzed fetal bovine serum (Invitrogen). Cells were detached with 0.05% trypsin (Sigma).

**Transfection**

SKBR3 or MCF7-R cells were plated in 35-mm plates. PPRHs were mixed with N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methysulfate (DOTAP, Roche) at the appropriate oligonucleotide–DOTAP molar ratio (1:10–1:100) for 15 min at room temperature before lipofecting the cells.

**MTT Assay**

SKBR3 or MCF7-R cells (10,000) were plated in 35-mm plates. MTT assays using PPRHs against dhfr were performed in −GHT medium. After 7 days, 500 μg of MTT (Sigma) and 5.6 mg of succinate (Sigma) were added and allowed to react for 3 h at 37 °C before the addition of the solubilization reagent (0.57% acetic acid and 10% SDS in dimethyl sulfoxide). Cell viability was measured at 570 nm in a WPA S2100 Diode Array Spectrophotometer.

**mRNA Analysis**

72 h after PPRH transfection, total RNA from 30,000 SKBR3 or MCF7-R cells, was extracted using the Ultra-spec™ RNA Kit (Biotec), according to the manufacturer’s specifications. The amount of RNA was determined by measuring its absorbance (260 nm) at 25 °C in a Nanodrop ND-1000 spectrophotometer.

cDNA was synthesized in a 20-μl reaction mixture containing 0.2–0.5 μg of total RNA, 125 ng of random hexamers (Roche), 10 mM dithiothreitol, 20 units of RNasin (Promega), 0.5 mM dNTPs (AppliChem), 4 μl of 5× reverse transcriptase buffer, and 200 units of Moloney murine leukemia virus reverse transcriptase (RT) (Invitrogen). The reaction mixture was incubated at 37 °C for 1 h. Three μl of the cDNA mixture was used for PCR amplification by real time.

*Real Time-PCR—* The reaction was performed using the ABI-PriSm 7000 Sequence Detection System (Applied Biosystems). For dhfr, ugt1a10, and s100A4 (the two latter were used when checking for PPRH specificity), the reaction was carried out in a final volume of 20 μl, containing 1× TaqMan Universal PCR Mastermix (Applied Biosystems), 1× TaqMan probe (Applied Biosystems), 3 μl of cDNA and H₂O. For telomerase ( tert) and survivin (birc5), a SYBR Green PCR was performed in a final volume of 20 μl, containing 1× SYBR Universal PCR Mastermix (Applied Biosystems), 0.25 μM of reverse and forward primers (Ecogen), 3 μl of cDNA and H₂O. Either for the TaqMan or the SYBR Green reactions, the adenosylphosphoribosyl transferase (APRT) mRNA was used to normalize the results. The primer sequences were: Tert-Fw, 5’-AGGAGCTGAC-GTGGGAAGATG-3’, Tert-Rv 5’-GCTGCAGCAGCTACACTC-3’; Surv-Fw, 5’-AGCCAGATGACGACCCATAG-3’, Surv-Rv, 5’-CACAGGAAGGCTGGTGGCAC-3’; Aprt-Fw, 5’-GGCACTGTTGGACACGGGAT-3’, Aprt-Rv, 5’-AGAGTG-GGGGACTGACTTC-3’. PCR cycling conditions were 2
TABLE 1
Sequence of the PPRHs used in this study and their target sequences

| Name                  | Sequence                                                                 |
|-----------------------|--------------------------------------------------------------------------|
| dhfr target 1 template| Target sequence intron 3, reverse strand: 5’-...AGGAGGGAGGGAGGGAGGAGAG-3’ |
| HpdI3-B               | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                           |
| HpdI3-T               | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                           |
| HpdI3-BT              | 5’-GAAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| HpdI3-WC              | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| HpdI3-NH              | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| HpdI3-Sc              | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| HpdI3-C               | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| HpdI3-sf              | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| dhfr target 2 template| Target sequence intron 3, reverse strand: 5’-...AGGAGGGAGGGAGGGAGGAGG-3’ |
| HpdI3-misTA           | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| HpdI3-misTG           | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| HpdI3-misTT           | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| dhfr target 3 template| Target sequence intron 3, reverse strand: 5’-...AGGAGGGAGGGAGGGAGGAGG-3’ |
| HpdI3-misCA           | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| Tert template         | Target sequence intron 8, reverse strand: 5’-...GAGGAGGGAGGGAGGGAGGAGG-3’ |
| HptI8-B               | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| HptI8-WC              | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| HptI8-Sc              | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| birc5 template        | Target sequence exon 3, reverse strand: 5’-...GAGGAGGGAGGGAGGGAGGAGG-3’ |
| HpsPr-B               | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| HpsPr-Sc              | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |
| HpsPr-WC              | 5’-GAGGAGGGAGGGAGGGAGGAGGAG-3’                                          |

FIGURE 1. Binding of PPRHs to polypurine/polypyrimidine target sequence-1 in the dhfr gene. A, schematic representation of the binding of the PPRHs to the dsDNA dhfr target-1 in intron 3 of the dhfr gene; B, binding of the different PPRHs to 20,000 cpmp of [32P]-dhfr target-1. Dpx, duplex free target; Hp-B, hairpin HpdI3-B (blunted); Hp-T, hairpin HpdI3-T (tailed); Hp-BT, hairpin HpdI3-BT (tailed and blunted); Hp-WC, hairpin HpdI3-WC (Watson-Crick); Hp-NH, HpdI3-NH (no Hoogsteen) and Hp-Sc (scrambled). Arrows indicate the bands corresponding to the different species (duplex or triplex) in the image obtained by PhosphorImaging.

min at 50 °C, 10 min denaturation at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The mRNA amount of the target gene, normalized to APRT, was given by the \( \Delta \Delta C_T \) method, where \( C_T \) is the threshold cycle indicating the fractional cycle number at which the amount of amplified mRNA reached threshold.
Western Analysis

MCF7-R cells were used to detect DHFR protein. 30,000 MCF7-R were plated in 35-mm plates and treated with PPRHs. 72 h after transfection, total protein extracts were obtained after scraping the cells in Lysis buffer (50 mM HEPES, 0.5 M NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100, supplemented with Protease Inhibitor Mixture by Sigma). The extracts were maintained at 4 °C for 1 h with vortexing every 15 min. Cell debris was removed by centrifugation (10,000 g for 10 min). Protein concentrations were determined by the Bio-Rad protein assay (based on the Bradford method, using bovine serum albumin as a standard (Sigma)).

Total protein cell extracts (35 µg) were electrophoresed on SDS-12% polyacrylamide gels, and transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore) using a semidry electroblotter. The membranes were probed with antibodies against DHFR (Davids Biotechnologie) and tubulin (Calbiochem) to normalize the results. Signals were detected by secondary horse-radish peroxidase-conjugated antibody (1:2500 dilution) (GE Healthcare) and enhanced chemiluminescence, as recommended by the manufacturer (GE Healthcare). Quantification was performed using ImageQuant 5.2 (GE Healthcare).

DHFR Activity Assay

72 h after PPRH transfection, DHFR activity was determined by incorporation of radioactive deoxyuridine to cellular DNA (24), with the following modifications: 10,000 SKBR3 cells were plated in 35-mm plates with 1 ml of F-12 medium (Ham’s F-12 medium lacking thymidine). Twenty-four hours before collecting the cells, 2 µCi of 6-[3H]deoxyuridine were added. The cells were then rinsed twice in 2 ml of phosphate-buffered saline and lysed in 100 µl of 0.1% SDS. The lysate was collected on Whatman 31ET papers (2.5 × 2.5 cm), which were immediately placed onto ice-cold 66% ethanol containing 250 mM NaCl, to precipitate DNA. After 3 washes in the same solution, the papers were dried and tritium was counted using liquid scintillation mixture (CytoScint, MP).

Flow Cytometry

The uptake of a fluorescein-labeled PPRH was monitored by flow cytometry on a Beckman Coulter Epics XL cytometer. 100,000 SKBR3 cells were plated in 35-mm dishes with Ham’s F-12 medium and transfected with HpdI3-B fluoresceinated in the 5’ end (HpdI3-BF, Sigma). Cells were collected in 500 µl of ice-cold Ham’s F-12 medium, centrifuged at 1,000 × g at 4 °C.
for 5 min, and washed twice in phosphate-buffered saline. The pellet was finally resuspended in 500 μl of phosphate-buffered saline. Propidium iodide (10 μg/ml, Sigma) was added and cells were kept on ice until the cytometric analysis. The percentage of fluorescein-positive cells after subtraction of the IP-positive fraction (dead cells) was used to express the cellular uptake of fluorescein-positive cells after subtraction of the IP-positive fraction (dead cells) was used to express the cellular uptake of fluorescein-positive cells after subtraction of the IP-positive fraction.

**Statistical Analysis**

Data are presented as mean ± S.E. Statistical analysis was performed using Student’s t test using SPSS 13.0 software for Mac OS X. Results were considered significant if \( p < 0.05 \) (*), \( p < 0.01 \) (**), or \( p < 0.005 \) (***)

**RESULTS**

**Design of PPRHs**—We searched for polypurine stretches within the human dihydrofolate reductase (dhfr), telomerase (tert), and survivin (birc5) genes, with a length of at least 20 or more bases to avoid PPRH unspecificity. Three polypurine stretches were selected in the template strand of the dhfr gene (Table 1). All sequences were located within intron 3. Dhfr target-1 had no purine interruptions in the polypurimidine stretch, whereas dhfr target-2 and -3 contained one purine interruption each, A and G, respectively. Template-PPRH Hp-B (blunted) was designed to bind the polypuriminidine reverse strand of the double helical DNA target by Watson-Crick base pairing. Additionally, PPRHs with the Watson-Crick domain extended with a tail (Hp-T), with both domains extended (Hp-BT, blunted and tailed) or with the loop on the contrary side (Hp-C) of the hairpin structure, were used. As negative control PPRHs, oligonucleotides bearing either: scrambled sequences (Hp-Sc) or intramolecular Watson-Crick bonds instead of Hoogsteen bonds (Hp-WC) were used. The effect of structures without the ability to form intramolecular reverse Hoogsteen bonds (No Hoogsteen, Hp-NH) but maintaining the target-binding domain was also explored. An antisense oligonucleotide (HATNL-24, 5’-gatGcAGtttAGcGGAAccAAccaT-3’), bearing the phosphothioate modification (at the 3’ of the bases indicated in lowercase) directed against the translational start of the dhfr gene, was used to test the responsiveness of cells when DHFR mRNA was targeted (25). The selected template-PPRH against the telomerase gene was located in intron 8, and contained three purine interruptions. Regarding survivin, template-PPRHs were targeted toward exon 3 and the promoter region, and both contained three purine interruptions. To check for the specificity of all these sequences BLAST analyses were performed. The PPRHs sequences are listed in Table 1 and their structures and targets are depicted in Figs. 1A, 6, A and B, 7A, and 8A.

**Binding of PPRHs to Polypurine/Polypyrimidine dsDNA**—The ability of PPRHs to bind their target sequence was tested by gel-shift assays. In Fig. 1B, the bindings of template-PPRHs to a duplex DNA containing the dhfr target 1 sequence are shown. We can observe a retardation of the duplex incubated with the three template-PPRHs (Hpdl3-B, Hpdl3-T, and Hpdl3-BT) as compared with the probe alone. The negative controls Hpdl3-WC and Hpdl3-Sc did not cause any shift in mobility, indicating a lack of union between the PPRH and the duplex, whereas Hpdl3-NH is responsible for a mobility retardation similar to that experimented by Hpdl3-B. Binding specificity was tested by the addition of 2 μg of tRNA to the incubation
reaction. Although template-PPRH can bind to ssDNA (data not shown), this molecular species cannot be found in the cellular context.

**Cytotoxicity of dhfr PPRHs**—We analyzed the effect of PPRHs on SKBR3 survival (Fig. 2A). Template-PPRH HpdI3-B was lipofected with DOTAP, provoking 70% of cytotoxicity after 1 week of incubation, whereas the aODN HATNL-24, used for comparative purposes, produced 50% of cell death. DOTAP alone, at the concentration used to transfect PPRHs, HpdI3-B without DOTAP and the negative controls HpdI3-WC and HpdI3-Sc did not affect cell survival. HpdI3-NH showed a 43% cytotoxicity acting just as antisense. Increasing PPRH:DOTAP ratios in combination with decreasing HpdI3-B concentrations ranging from 500 to 100 nm with 10 μM DOTAP were tested to study the optimal PPRH:DOTAP relation (Fig. 2B). The best results were obtained with 100 nm HpdI3-B, 1:100 PPRH:DOTAP molar ratio, producing 87% cell mortality.

Modifications in the PPRH structure were also studied (Fig. 2C). HpdI3-C is equivalent to HpdI3-B but with the loop on the other side. This change of orientation did not cause an increment in activity. HpdI3-T (tailed in the Watson-Crick domain) and HpdI3-BT (tailed and blunted) bear a single or double strand elongated sequence, respectively (see Table 1). Both produced less cytotoxicity than HpdI3-B.

The time needed for HpdI3-B to develop its cytotoxic action was also studied. After treatment, PPRHs and liposome were removed by changing the medium at time intervals of 24 h for 4 days. Incubation of cells with HpdI3-B for 24 h already provoked high cell mortality, achieving 99% cytotoxicity when the cells were incubated with HpdI3-B for 96 h (Fig. 2D).

**Effects of PPRHs on DHFR Activity**—The remaining DHFR activity in SKBR3 cells after 72 h of treatment with PPRHs was measured by the [3H]deoxyuridine assay. As shown in Fig. 3A, template-PPRH HpdI3-B was able to decrease DHFR activity in a dose-dependent manner, causing a drop in activity of about 60% at the concentration of 100 nm, using a 1:100 PPRH:DOTAP molar ratio. Transfection with DOTAP or with the negative control HpdI3-WC did not affect significantly DHFR activity, whereas HpdI3-NH caused a 47% decrease in activity.

**Effects of PPRHs on DHFR mRNA Levels**—DHFR mRNA levels were determined by RT–real time-PCR 72 h after incubation with different PPRHs. HpdI3-B provoked a dose-dependent reduction in DHFR mRNA levels, reaching almost a 50% decrease upon cells lipofection with a concentration of 100 nm (Fig. 3B). Both HATNL-24 (aODN) and HpdI3-NH (No Hoogsteen) were also able to decrease DHFR mRNA. DOTAP alone and the negative control HpdI3-WC (Watson-Crick) and HpdI3-Sc (Scrambled) did not produce a reduction in DHFR mRNA.

**Effects of PPRHs on Methotrexate-resistant Cells**—The possible therapeutic action of PPRHs against dhfr was tested in breast cancer cells resistant to 10⁻⁶ M methotrexate. MCF7-R cells showed a 70% cell mortality after transfection with template-HpdI3-B, 100 nm 1:100 PPRH:DOTAP molar ratio, whereas no significant cytotoxicity was observed with the antisense oligonucleotide HATNL-24 (Fig. 4A). HpdI3-NH also produced a noticeable decrease in cell survival (52%). In this case, DOTAP alone and the negative control HpdI3-WC provoked a certain degree of cytotoxicity. For comparison purposes, when using a siRNA against DHFR (siRNA-DHFR: 5′-AAUGAGCUCCUUGUGGAGGT-3′) at 100 nm, the cytotoxic effect was only 30%.

DHFR protein levels in MCF7-R cells were analyzed by Western blot using tubulin to normalize the results. A representative image of DHFR protein levels after PPRH treatment is shown in Fig. 4B. DHFR protein levels, 30,000 MCF7-R cells were incubated with DOTAP alone (30 μM), or in combination with 300 nM of the indicated PPRHs. Three days after transfection, DHFR protein levels were determined by Western blot using tubulin to normalize the results. A representative image of DHFR and tubulin levels after PPRH treatment is shown. C, DHFR mRNA levels, 30,000 MCF7-R cells were incubated with 30 μM DOTAP alone, aODN HATNL-24, and the indicated PPRHs. Three days after transfection, DHFR mRNA levels were determined by RT–real time-PCR using the signal corresponding to APRT mRNA to normalize the results. Data represent the mean ± S.E. of at least four experiments. *p < 0.02; **p < 0.01; ***p < 0.005.

**FIGURE 4.** Effects of template-PPRHs directed against the dhfr gene in MCF7 cells resistant to 10⁻⁶ M methotrexate. A, cytotoxicity. 10,000 MCF7-R cells were plated in DHFR selective medium (−GHT). Cells were incubated with hairpins HpdI3-B, HpdI3-NH, or HpdI3-SC at the indicated concentrations and 10 μM DOTAP. A week after treatment an MTT assay was performed. Data represent the mean ± S.E. of at least four experiments. B, DHFR protein levels. 30,000 MCF7-R cells were incubated with DOTAP alone (30 μM), or in combination with 300 nm of the indicated PPRHs. Three days after transfection, DHFR protein levels were determined by Western blot using tubulin to normalize the results. A representative image of DHFR and tubulin levels after PPRH treatment is shown. C, DHFR mRNA levels, 30,000 MCF7-R cells were incubated with 30 μM DOTAP alone, aODN HATNL-24, and the indicated PPRHs. Three days after transfection, DHFR mRNA levels were determined by RT–real time-PCR using the signal corresponding to APRT mRNA to normalize the results. Data represent the mean ± S.E. of at least four experiments. *p < 0.05; **p < 0.01; ***p < 0.005.
levels (48%) was observed when cells were treated with template-HpdI3-B, 300 nM, whereas HpdI3-NH just caused a 30% decrease. In contrast, when treating cells with either DOTAP alone or the template negative control Hp-WC, no significant decrease in the protein levels was observed.

DHFR mRNA levels in MCF7-R cells were determined 72 h after PPRH transfection and analyzed by RT-real time-PCR. Template-PPRHs HpdI3-B and HpdI3-NH produced a significant drop of DHFR mRNA (56% of the control) (Fig. 4C), whereas DOTAP alone, aODN HATNL-24, and the negative control HpdI3-WC did not cause significant changes in DHFR mRNA levels.

**PPRH Uptake and Stability**—To analyze the intracellular amount of PPRHs after transfection, the fluorescence levels of cells transfected with a fluorescent PPRH was analyzed by flow cytometry at different times after transfection. The mean fluorescence rose gradually to reach its maximum value at 48 h. From that point the mean fluorescence started to decrease (Fig. 5A).

The half-life of fluorescent PPRH was determined by performing a wash out assay after 24 h of transfection. A slow decrease in fluorescence in cells during the first 5 days was observed, followed by a marked drop between the fifth and the sixth day. The half-life was about 5 days (Fig. 5B).

**Effects of PPRHs on Targets Containing Purine Interruptions**—In the case of purine interruptions in the target sequence, we explored the best nucleotide to be used in the template-PPRH to compensate for the presence of the interruption. PPRHs with the same sequence except for the nucleotide matching the purine interruption were tested (Fig. 6, A and B). Two different target sequences in the *dhfr* reverse DNA strand containing purine interruptions were found, one containing an A (target-2) and the other a G (target-3). For the reverse DNA target sequence that contained an A, three template-PPRHs were designed: HpdI3-MisTA (containing an A in front of the A of the reverse strand), HpdI3-MisTG (containing a G in front of the A of the target strand), and HpdI3-MisTT (containing a T in front of the A of the reverse strand). All PPRHs bound to ssDNA-Rev, which confirmed the ability of PPRHs to form triple helices with polypyrimidine ssDNA. As expected, no binding was observed between PPRHs and the polypurine single strand DNA-For (Fig. 6, A and B). The differences in triple formation among these PPRHs and their dsDNA target sequence were quantified by measuring the amount of triplex formed. HpdI3-MisTT had the weakest binding affinity to dsDNA (28% binding compared with the probe alone), whereas HpdI3-MisTA and HpdI3-MisTG had similar affinity (37 and 42%, respectively to dsDNA) (Fig. 6A).

Regarding the *dhfr* reverse DNA target sequence containing a G, we used the template-PPRH HpdI3-MisCA (containing an A in front of the G of the target strand) because previous studies in our group revealed that it produced the most stable binding. The binding affinity of HpdI3-MisCA with its corresponding duplex target sequence (dsDNA) was 72% (Fig. 6B), in agreement with previous results obtained in our laboratory (17) that pointed out that adenine was the best option for a guanine interruption.

The cytotoxic effect of PPRHs targeting sequences bearing one-base interruption was analyzed in SKBR3 cells. The selection of the PPRHs HpdI3-MisTA, HpdI3-MisTG, and HpdI3-MisCA was made according to the results obtained in the binding assays (Fig. 6, A and B). As we can observe in Fig. 6C, HpdI3-MisTA had a higher cytotoxicity than HpdI3-MisTG, and similar to that caused by HpdI3-MisCA. However, all the PPRHs containing an interruption showed lower cell mortality than HpdI3-B, without interruptions in the target strand.

**Effects of Telomerase PPRHs**—PPRHs targeting the telomerase gene were designed following the same strategy as with the *dhfr* gene (Fig. 7A). HptI8-B was directed to a 30-nt polypurine/polypyrimidin stretch in the intron 8 and bore 3-purine interruptions. The cytotoxicity caused by a 100 nM HptI8-B, 1:100 PPRH:DOTAP molar ratio, on SKBR3 cells was 90% (Fig. 7B). No cell mortality was observed when cells were transfected with
DOTAP alone or with the negative controls (HptI8-WC and HptI8-Sc).

Telomerase mRNA levels were analyzed by RT-real time-PCR, observing a noticeable decrease in its expression when SKBR3 cells were treated with 100 nM HptI8-B (Fig. 7). The negative controls HptI8-WC and HptI8-Sc did not cause a significant change in telomerase mRNA levels.

Effects of Survivin PPRHs—The antiapoptotic survivin gene was the target of two PPRHs, one directed toward exon 3, HpsE3-B, and the second toward the promoter region, HpsPr-B (Fig. 8A). HpsE3-B was directed to a 29-nt polypurine/polyymidnine stretch in the exon 3 and bore 3-purine interruptions, and HpsPr-B was directed to a 26-nt polypurine/polyymidnine stretch in the promoter region and contained 3-purine interruptions. HpsPr-B at a 1 μM 1:10 PPRH:DOTAP molar ratio caused 96% of cytotoxicity, whereas HpsE3-B at 1 μM 1:10 DOTAP produced 27% of cell mortality (Fig. 8B). The negative controls for HpsPr-WC and HpsPr-Sc did not cause a significant decrease in survivin mRNA levels.

PPRH Specificity—The specificity of the designed PPRH was checked both by BLAST analysis and using negative controls (Hp-WC and Hp-Sc). Additionally, we also tested whether PPRHs unspecifically decreased mRNA levels of unrelated genes by RT-real time-PCR: dhfr, telomerase, survivin (when they were not their target), ugt1a10 and s100a4. As observed in Table 2, PPRHs did not produce a significant mRNA decrease of non-targeted genes.

DISCUSSION

We previously described the ability of PPRHs to bind double-stranded DNA forming triplex molecules (17). Two strands
come from the hairpin, linked by intramolecular Hoogsteen bonds, and the third one from the dsDNA, which binds to the hairpin by Watson-Crick bonds. In the present study we tested whether polypurine reverse Hoogsteen hairpins had an effect on gene expression. Thus, we developed different PPRHs that bind to the template DNA strand and incubated them with mammalian cells to analyze the effect on the different stages of gene expression and cytotoxicity.

As a model to test the efficacy of template-PPRHs on decreasing gene expression, we used the \textit{dhfr} gene. Optimal \textit{dhfr} polypyrimidine stretches, the target sequences of PPRHs, were found in introns. This is not surprising because these stretches are found predominantly in introns, promoters, and with less probability in exons. However, these polypyrimidine stretches are found in the genome with higher frequency than predicted by the random assembly of the four bases; for this reason they are thought to have a regulatory activity on genes (18, 19). Almost all genes contain this type of sequences, implying that PPRHs could be used to target a great number of genes.

We found that the most active PPRH, in binding and cytotoxicity, had a blunted hairpin structure (HpdI3-B). The PPRH with an extended fragment of the Watson-Crick binding domain lacking Hoogsteen unions (tailed, HpdI3-T) was less active. This may be explained by the fact that intramolecular Hoogsteen bonds in the whole hairpin stabilize triplex unions (16). It was also found that a longer PPRH (32 nt, HpdI3-BT), although theoretically more sequence specific, had less activity.

HpdI3-NH reveals the effect of Hoogsteen bonds in PPRH activity, this hairpin has the ability to bind to the polypyrimidine target sequence but it cannot make Hoogsteen bonds. This no Hoogsteen hairpin is active in a lower degree than PPRHs, as it resembles an aODN with a hanging tail. Therefore, the binding of a double strand target by triplex formation with PPRHs is more stable than that produced by oligonucleotides through Watson-Crick bonds (17).

The frequency of finding polypyrimidine regions lacking purine interruptions is low; for this reason we studied the ways to minimize the instability that these interruptions produce during triplex formation. We found that adenine was the best base to place in the PPRH sequence to pair with a purine interruption in the polypyrimidine target sequence. These results are in agreement with previous studies made with guanine interruptions, in which an adenine was also the best nucleotide to stabilize triplex formation (17).

Next, we analyzed the effects of PPRHs on mRNA and protein levels, enzymatic activity, and the resultant cytotoxicity. Our results show that PPRHs against the \textit{dhfr} gene decrease DHFR mRNA levels, and as a consequence, induce a decrease in DHFR protein levels and enzymatic activity.
These results are consistent with the mechanism of action of the PPRHs. They bind to polypyrimidine stretches present into the DNA and, as a consequence they interfere with transcription, as revealed by the mRNA decrease. Therefore, template-PPRHs would share with TFOs an equivalent mechanism of action. Although the greatest inhibition of DNA transcription by triplexes is found when the target duplex is in the gene promoter (18), the inhibition of DNA transcription by triplexes can occur by means of the inhibition of mRNA elongation (26, 27) as it would be the case when the target sequence is intronic.

**TABLE 2**

PPRH specificity

|          | dhfr | Telomerase | Survivin | UGT1A10 | S100A4 |
|----------|------|------------|----------|---------|--------|
| Control  | 100  | 100        | 100      | 100     | 100    |
| HpdI3-B  | 142.6 | 11.5       | 115      | 7.9     | 99.3   |
| HpI8-B   | 94   | 19.3       | 111.1    | 19.6    | 169.4  |
| HpsPr-B  | 78.6 | 5          | 108.2    | 28.7    | 128.7  |
| HpsPr-WC |       |            |          |         |        |
| HpsPr-Sc |       |            |          |         |        |

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PPRHs display a series of advantages: (i) they work at concentrations of 100 nM (similar to that used for siRNAs). (ii) They are very active in producing final cytotoxicity, more than aODNs (HATNL-24), siRNAs (siRNA-DHFR), and PPRH structures lacking Hoogsteen bonds (HpdI3-NH). (iii) PPRHs present a remarkable increase in stability (half-life of 4.9 days compared with the 2 days for modified aODNs) (28, 29), without the need for modified nucleotides. (iv) PPRHs are easy to synthesize and inexpensive, as they are very stable non-modified single-stranded DNA oligonucleotides, with no special handling requirements in contrast to siRNAs.

The specificity of PPRHs for their target sequences was monitored by the use of negative controls (Hp-WC and Hp-Sc), BLAST analysis, and the study of mRNA decrease of unrelated genes when cells were transfected with the different PPRHs. Hp-WC is a negative control because despite having the target binding sequence, its intramolecular complementary strand makes Watson-Crick bonds instead of Hoogsteen bonds, producing a closed hairpin structure unable to form triplex with the target sequence. Hp-Sc, an oligonucleotide bearing scrambled sequences, also demonstrates PPRHs specificity of action.

We also proved that PPRHs are not only active toward the dhfr gene, but also against other genes containing polypyrimidine-rich regions. telomerase gene has an intronic polypyrimidine sequence that contains three purine interruptions. The PPRH against telomerase showed a noticeable cytotoxicity after replacing these interruptions with three adenines.
regions, either in the exonic sequence or the promoter. The regulatory activity of polypyrimidine tracts would be reinforced by the result that exon-PPRH scarcely caused cell cytotoxicity, whereas PPRH toward the promoter showed a remarkable cell death.

In addition, we present the efficacy of PPRHs directed toward dhfr in breast cancer cells that have developed resistance to the chemotherapeutic drug methotrexate. This compound is a competitive inhibitor that blocks folic acid binding to DHFR (34, 35). MCF7-R cells able to survive at methotrexate concentrations of 10^{-6} M died when transfected with PPRHs that knocked down the dhfr gene. This approach to target genes responsible for resistance to chemotherapy, or to use PPRHs that target crucial genes involved in cancer processes, as telomerase and survivin, turns PPRHs into potential gene therapy tools.

In summary, we describe the use of PPRHs in cultured cells and their biological effect. PPRHs cause a decrease in mRNA levels that lead to a cytotoxic effect when essential genes in cell growth are targeted. PPRHs could represent a new tool to inhibit gene expression in cancer therapy.

REFERENCES
1. Chan, P. P., and Glazer, P. M. (1997) J. Mol. Med. 75, 267–282
2. Giovannangeli, C., and Häliene, C. (1997) Antisense Nucleic Acid Drug Dev. 7, 413–421
3. Casey, B. P., and Glazer, P. M. (2001) Prog. Nucleic Acids Res. Mol. Biol. 67, 163–192
4. Giovannangeli, C., and Haëliene, C. (1997) J. Mol. Med. 75, 2593–2613
5. Ryan, K., and Kool, E. T. (1998) Chem. Biol. 5, 59–67
6. Sorensen, J. J., Nielsen, J. T., and Petersen, M. (2004) Nucleic Acids Res. 32, 6078–6085
7. Brunet, E., Corgnali, M., Perrouault, L., Roig, V., Asseline, U., Sorensen, M. D., Babu, B. R., Wengel, J., and Giovannangeli, C. (2005) Nucleic Acids Res. 33, 4223–4234
8. Giovannangeli, C., Monteny-Garestier, T., Rougee, M., Chassignol, M., Thoung, N. T., and Helene, C. (1991) J. Am. Chem. Soc. 113, 7775–7777
9. Chasin, L. (1985) Mol. Cell. Biol. 5, 431–432
10. Faucon, B., Mergny, J. L., and Helene, C. (1996) Nucleic Acids Res. 24, 3181–3188
11. Mills, M., Arimondo, P. B., Lacroix, L., Garestier, T., Klump, H., and Mergny, J. L. (2002) Biochemistry 41, 357–366
12. Aviñó, A., Cubero, E., González, C., Eritja, R., and Orozco, M. (2003) J. Am. Chem. Soc. 125, 16127–16138
13. Coma, S., Noe, V., Eritja, R., and Ciudad, C. J. (2005) Oligonucleotides 15, 269–283
14. Faucon, B., Bhuyan, P., Capodici, J., and Weissman, D. (2004) J. Immunol. 172, 988–993
15. Vasquez, K. M., Wensel, T. G., Hogan, M. E., and Wilson, J. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4199–4203
16. Felsenfeld, G., and Rich, A. (1957) Biochim. Biophys. Acta 26, 457–468
17. Hewett, P. W., Daft, E. L., Laughton, C. A., Ahmad, S., Ahmed, A., and Murray, J. C. (2006) Mol. Med. 12, 8–16
18. Gonin, J. R., de la Cruz, X., and Orozco, M. (2004) Nucleic Acids Res. 32, 354–360
19. Gonin, J. R., Vaquerizas, J. M., Dopazo, J., and Orozco, M. (2006) BMC Genomics 7, 63
20. Postel, E. H., Flint, S. J., Kessler, D. J., and Hogan, M. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8227–8231
21. Kim, H. G., Reddoch, J. F., Mayfield, C., Ebbinghaus, S., Vigneswaran, N., Thomas, S., Jones, D. E., Jr., and Miller, D. M. (1998) Biochemistry 37, 2299–2304
22. Faria, M., Wood, C. D., Perrouault, L., Nelson, J. S., Winter, A., White, M. R., Helene, C., and Giovannangeli, C. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3862–3867
23. Karpilow, J., Marshall, W. S., and Khvorova, A. (2006) Cold Spring Harb. Symp. Quant. Biol. 71, 453–464
24. Peyman, A., Helsberg, M., Kretzschmar, G., Mag, M., Ryte, A., and Uhlmann, E. (1997) Antiviral Res. 33, 135–139
25. Faria, M., Wood, C. D., Perrouault, L., Nelson, J. S., Winter, A., White, M. R., Helene, C., and Giovannangeli, C. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3862–3867
26. Manche, L., Green, S. R., Schmedt, C., and Mathews, M. B. (1992) J. Biol. Chem. 267, 16274–16282
27. Chasin, L. (1985) Mol. Cell Biol. 5, 59–67
28. Campbell, J. M., Bacon, T. A., and Wickstrom, E. (1990) J. Biochem. Biophys. Methods 20, 259–267
29. Peyman, A., Kretzschmar, M., Karpilow, J., Marshall, W. S., and Khvorova, A. (2006) Cold Spring Harb. Symp. Quant. Biol. 71, 453–464
30. Sorensen, J. J., Nielsen, J. T., and Petersen, M. (2004) Nucleic Acids Res. 32, 6078–6085
31. Brunet, E., Corgnali, M., Perrouault, L., Roig, V., Asseline, U., Sorensen, M. D., Babu, B. R., Wengel, J., and Giovannangeli, C. (2005) Nucleic Acids Res. 33, 4223–4234
32. Giovannangeli, C., Monteny-Garestier, T., Rougee, M., Chassignol, M., Thoung, N. T., and Helene, C. (1991) J. Am. Chem. Soc. 113, 7775–7777
33. Kandimala, A. S., Agrawal, S., Venkataraman, S., and Saishekaran, V. (1995) J. Am. Chem. Soc. 117, 6416–6417
34. Aviñó, A., Morales, J. C., Frieden, M., de la Torre, B. G., García de la Torre, B., Géuimil, F., Gelpá, J. L., Orozco, M., González, C., and Eritja, R. (2002) Nucleic Acids Res. 30, 2609–2619
35. McGuire, J. J. (2003) Curr. Pharm. Des. 9, 2593–2613

PPRHs, A New Tool to Knock Down Gene Expression

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In summary, we describe the use of PPRHs in cultured cells and their biological effect. PPRHs cause a decrease in mRNA levels that lead to a cytotoxic effect when essential genes in cell growth are targeted. PPRHs could represent a new tool to inhibit gene expression in cancer therapy.