PolyPurine Reverse Hoogsteen hairpins for silencing expression of cancer target genes in human cells

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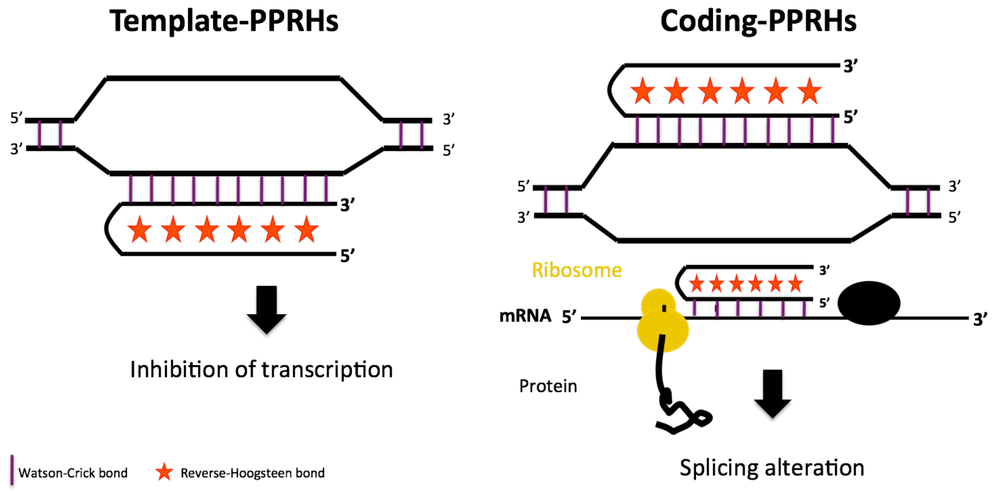
ABSTRACT

PolyPurine Reverse Hoogsteen hairpins (PPRHs) are molecules formed by two antiparallel polypurine strands bound by intramolecular Hoogsteen bonds linked by a pentathymidine loop. These hairpins bind to their target DNA sequence by Watson–Crick bonds producing a displacement of the 4th strand and a specific reduction of gene expression. PPRHs have the advantage over other silencing molecules such as siRNAs because of their stability, economy of synthesis and lack of immunogenicity. Previously, we targeted genes involved in metabolism (DHFR), proliferation (mTOR), DNA topology (TOP1), lifespan and senescence (telomerase), apoptosis (survivin, BCL2), transcription factors (MYC), proto-oncogenes (MDM2), and immunotherapy (CD47/SIRPα). Presently, we designed PPRHs against the antiapoptotic gene Mcl1, genes involved in replicative stress (Wee1, CHk1) and the tandem PD1/PDL1 for immunotherapy.
Three different PPRHs, against the promoter, exon 1 and intron 2 of MCL1, caused a decrease in survival in HeLa, PC3, HepG2 and MCF7 cancer cells of over 80% at 100 nM, and an increase in apoptosis.PPRHs against the promoter and intron of WEE1 or against intron 1 of CHK1 showed a high decrease in cell viability in HeLa, SKBR3, MCF7 and PC3 cells and an increase in apoptosis.
Various PPRHs targeting different regions of both PD-1 (promoter, intron 4 and exon 4) and PD-L1 (intron 1 and intron 2) genes in different combinations provoked a decrease in cell viability of at least 55% in co-culture experiments. The most effective combination was the PPRH against intron 2 of PD-L1 with the PPRH against intron 4 of PD-1.

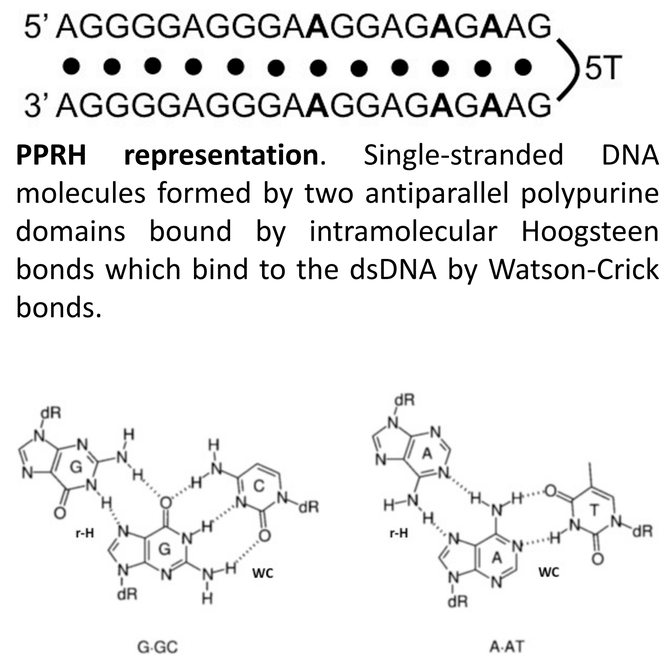
POLYPURINE REVERSE HOOGSTEEN HAIRPINS (PPRHs)

PPRHs advantages

- PPRHs work at concentrations of 100nM (similar to that used for siRNAs).
- PPRHs are very active in producing final cytotoxicity, and thus can be used as an alternative to aODNs and siRNAs.
- PPRHs present a remarkable increase in stability (in serum and cells) without the need to modify nucleotides.
- PPRHs are easy to synthesize and inexpensive, since they are non-modified single stranded DNA oligonucleotides, very stable, with no special handling requirements in contrast to siRNAs.
- PPRHs shows very little immunogenicity.
- PPRHs work both *in vitro* and *in vivo*.



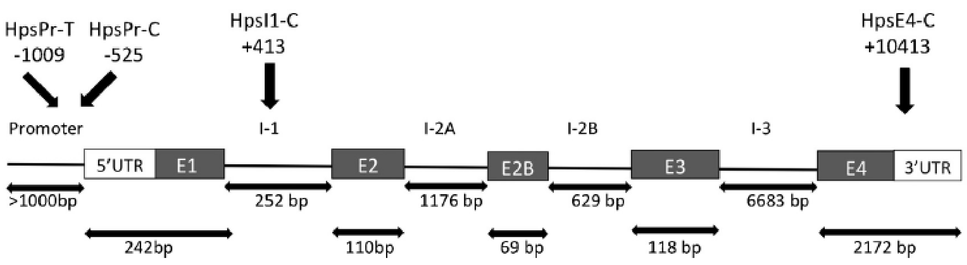
Mechanism of action. PPRHs bind in a sequence-specific manner to their target polipyrimidine sequence in the dsDNA by Watson-Crick bonds while maintaining the hairpin structure. Upon binding to genomic DNA, they produce strand displacement of the fourth strand thus inhibiting either transcription or splicing. Therefore, PPRHs mechanism of action is different than that of TFOs.



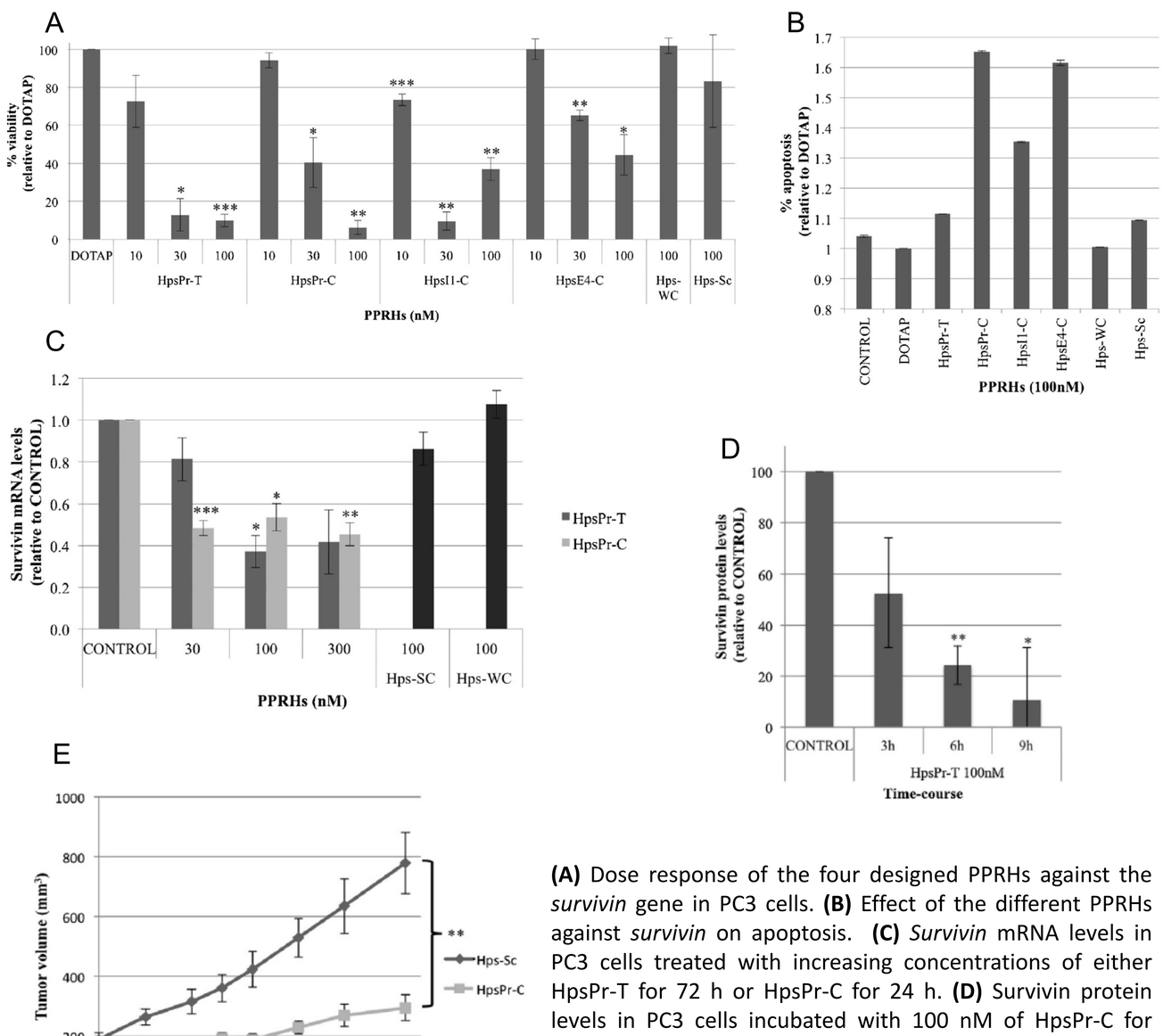
PPRH representation. Single-stranded DNA molecules formed by two antiparallel polypurine domains bound by intramolecular Hoogsteen bonds which bind to the dsDNA by Watson-Crick bonds.

Hoogsteen bonds. Guanidine in the middle can bind at the same time with either a C by Watson-Crick bonds and another G by Hoogsteen bonds, thus forming a triplex structure.

Example of target: *Survivin*



Survivin. Schematic representation of the *Survivin* gene structure and targeting with PPRHs.



(A) Dose response of the four designed PPRHs against the *survivin* gene in PC3 cells. **(B)** Effect of the different PPRHs against *survivin* on apoptosis. **(C)** *Survivin* mRNA levels in PC3 cells treated with increasing concentrations of either HpsPr-T for 72 h or HpsPr-C for 24 h. **(D)** Survivin protein levels in PC3 cells incubated with 100 nM of HpsPr-C for different periods of time. **(E)** Progression of tumor volumen throughout time when Hps-Sc (negative control) or HpsPr-C were administered by i.v. route twice a week in a *xenograft* model.

Validated cancer target genes of PPRHs in human cells

Compendium of the effects of PPRHs against different targets in cancer cell lines on cytotoxicity, apoptosis and mRNA and protein levels.

Targeted gene	Cell line	PPRH	Cytotoxicity (relative to control)	mRNA levels (relative to control)	Protein levels (relative to control)	Apoptosis (Fold-change relative to control)
DHFR	SKBR3	HpdI3-A-TA	86%	50%	60%	ND
	MCF7-R	HpdI3-B	71%	58%	60%	ND
telomerase	SKBR3	HptI8-B	90%	45%	ND	ND
survivin	PC3	HpsPr-T and HpsPr-C	90%	40%	20%	1.65
	HeLa		90%	ND	ND	ND
BCL2	MIA PaCa-2	HpBcl2E1-C	95%	55%	ND	2.2
	PC3		90%	55%	ND	2.0
	HCT 116		95%	60%	ND	7.8
TOP1	SKBR3	HpTopI2-T	95%	58%	ND	2.0
	MCF7		60%	55%	ND	2.5
	MDA-MB-468		85%	68%	ND	4.2
MDM2	SKBR3	HpMdmI7-T	50%	42%	ND	4.5
	MCF7		60%	65%	ND	4
	MDA-MB-468		85%	38%	ND	13.5
MYC	SKBR3	HpMycI1-T	85%	40%	ND	4.4
	MCF7		80%	43%	ND	3.5
	MDA-MB-468		95%	72%	ND	12.5
mTOR	HCT 116	HpTorPr-C	90%	59%	ND	4.0
CD47 and *SIRPα*	MCF7 and THP-1	HpCD47Pr-T and HpSIRPαI7-T	70%*	42%	40%	3.0*
				40%	20%	
MCL1	HeLa	HpMcl1Pr-C, HpMcl1-I2-C and HpMcl1-E1-C	95%	ND	ND	1.9
	PC3		95%			20.0
	MCF7		95%			ND
	HepG2		95%			ND
WEE1	HeLa	HpWEE1Pr-T, HpWEE1I5-C and HpWEE1E11-C	85%	ND	ND	4.5
	SKBR3		50%			ND
	MCF7		50%			ND
	PC3		80%			ND
CHK1	HeLa	HpCHK1I1-C and HpCHK1I1-T	80%	ND	ND	23.0
	SKBR3		70%			ND
	MCF7		90%			ND
	PC3		90%			ND
PD1 and *PDL1*	PC3 and THP-1	HpPD1E1-C HpPD1I4-C HpPD1Pr-T and HpPDL1I1-T HpPDL1I2-C	At least 80%	ND	ND	ND

For each targeted gene, cytotoxicity, apoptosis fold-change and mRNA and protein levels upon incubation with the corresponding PPRH is shown.

*In the immunotherapy experiments targeting CD47 in MCF-7 cells and SIRPα in THP-1 cells, the cytotoxicity and apoptosis fold-change corresponds to the treatment with both PPRHs in co-culture whereas the mRNA and protein levels correspond to each target separately.

Conclusion: PPRHs represent a new silencing tool to target genes related to cancer progression, drug-resistance and immunotherapy

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