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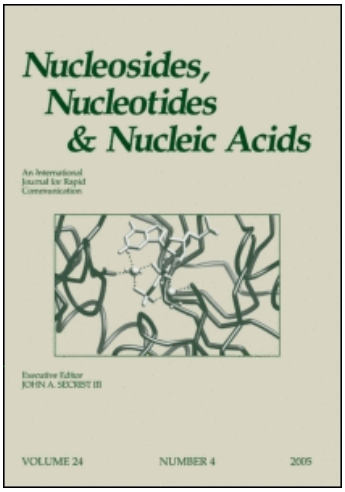

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ANTIGENE PROPERTY OF PNA CONJUGATED TO THE NUCLEAR LOCALIZATION SIGNAL PEPTIDE

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▫ *Peptide nucleic acid (PNA) is a DNA mimic with antigene properties. To enhance its capacity to enter in the cell and internalize in the nucleus, PNA has been conjugated to the nuclear localization signal (NLS) peptide, PKKKRKV. PNA-NLS conjugates form stable hybrids with complementary DNA strands and poorly tolerate mismatched base pairing. Employed against cancer-associated genes, PNA-NLS exhibited a potent and specific antigene activity, suggesting exciting therapeutic approaches to cancer.*

Keywords NLS-Conjugated PNA, Mutated K-ras Gene, Antigene Activity, Pancreatic Cells

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INTRODUCTION

Peptide nucleic acid (PNA) is a synthetic DNA mimic with a non-charged polyamide backbone to which the nucleobases are attached through a methylene carbonyl linkage.[1] PNA is completely resistant to nucleases and proteases and forms very stable heteroduplexes with complementary DNA and RNA sequences.[2] For these properties, PNA is between the most interesting molecules so far proposed to downregulate gene expression in a sequence-specific manner. In general, unmodified, carrier-free PNA is poorly taken up by cultured cells, though in neurons[3] and leukemic KYO-1 cells[4] uptake of free PNA was observed. To enhance the delivery and to achieve an effective nuclear accumulation, PNA has been covalently linked to a number of peptides (Table 1). Among them, the conjugate in which PNA is linked to a peptide derived from the nuclear localisation signal (NLS), PKKKRKV, is of particular interest. This basic peptide was first shown to permit the transfer of SV40 large T antigen across the nuclear membrane[5] and to allow the nuclear delivery of proteins.[6] The capacity of PNA-NLS conjugates to penetrate cell membranes has been investigated by confocal laser microscopy. It has been reported that rhodamine-labeled PNA-NLS was able

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TABLE 1 Important Peptides that Have Been Tethered to PNA

Peptide	Type of peptide	Reference
PKKKRKV	NLS	[4,7,8,12]
RQIKIWFQNRRMKWKK	p-Antennapedia	[8,9]
NKFFKFFKFFK	Bacterial wall peptide	[15]
KKWKMRRNQPWVKVNR	Retro-inverso peptide	[3]
KKKK	Cationic	[16]
GWTLNSAGYLLGKINLKALAALAKKIL	Transportan	[17]

to accumulate in the nuclei of Burkitt's lymphoma cells, whereas the analogue PNA conjugated to a scramble peptide (KKVKPKR) remained in the cytoplasm.[7] Similarly, a fluorescein-labeled PNA-NLS was found to enter in leukemic KYO-1 cells and locate in both cytoplasm and nuclei.[4] A more sophisticated approach to PNA delivery was obtained designing modular conjugates in which the PNA was linked to a peptide containing both an import sequence to cross the plasma membrane and a compartment signal to internalize in the nucleus. An example is the conjugate in which the PNA is linked to NLS (for nuclear addressing) and to a 16-residue peptide derived from *Antennapedia* homeodomain ($pAntp_{43-58}$) as a membrane transporter.[8,9] Moreover, the $pAntp_{43-58}$ peptide is covalently linked to NLS via a redox-cleavage disulphide linkage to ensure that only the PNA is transported into the nucleus. Through this modular transport, the PNA is efficiently delivered to the cells, but no data are available yet on the antigene or antisense property of such constructs.

In vitro studies have showed that the binding of PNA to duplex DNA occurs via strand displacement: a mechanism requiring that the DNA double helix is not too stable.[10] However, in cultured cells, the binding of PNA to the genome appears less difficult than expected. In fact, the structural changes occurring in chromatin when is transcriptionally active favour the interaction between PNA and its genomic target. In addition, the hybridization to the duplex is kinetically favored in the presence of energy of supercoiling.[11] The first convincing paper on the antigene activity of PNA-NLS conjugate was reported by Boffa and coworkers.[7] They showed that a 17-mer PNA-NLS, complementary to a unique sequence located at the beginning of the second exon of the c-*myc* oncogene predominantly entered in the nuclei of BL cells, inducing a rapid downregulation of c-*myc* expression. Subsequently, the same authors demonstrated in the same Burkitt's lymphoma cells that a PNA-NLS complementary to a Eμ intronic sequence selectively blocked the expression of c-*myc* under Eμ control, but not the expression of other c-*myc* alleles.[12]

Recently, we have used an NLS-conjugate PNA to knockdown the K-*ras* oncogene in pancreatic adenocarcinoma cells. The majority of pancreatic carcinomas exhibit a mutation at codon 12 of K-*ras* and this mutation is associated to cell transformation.[13] To reduce the proliferation of adenocarcinoma cells we

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propose to downregulate the expression of the mutated K-*ras* gene. To this purpose we designed a 15-mer PNA fully complementary to the antisense strand of the K-*ras* genome containing codon 12, with the assumption that the PNA should bind to the target by a strand-invasion mechanism. The structures of the PNA conjugates employed in our study consists of a 15-mer PNA sequences whose C-*terminus* is tethered to NLS, while the N-*terminus* is tethered to fluorescein via a rink-amide linker. The conjugates were synthesised by adaptation of established solid-phase peptide protocols. A detailed description of the synthesis of the anti K-*ras* conjugates was previously reported.[4] However, for a comprehensive review of the synthetic strategies to obtain PNA-peptide conjugates, we remand to Ref. [14]. The thermodynamic stability of the PNA-DNA hybrids formed by the anti K-*ras* PNA-NLS (N-tggagctgatggcgt-NLS) with its DNA target (mutated K-*ras*) have been spectroscopically measured to be as high as 85°C. Since Panc-1 cells express all three ras genes: H-, N- and K-*ras* we addressed the question whether the designed anti K-*ras* PNA conjugate inhibited only the target K-*ras* gene and not also the other *ras* genes. The three target sequences of the ras genes are characterized by a high homology, in particular between N-*ras* and H-*ras*:

$5' - \text{acgccatcagctcca} - 3'$	K – ras(mutated)	target
$5' - \text{ac}\mathbf{a}\text{cc}\mathbf{g}\text{c}\mathbf{c}\mathbf{g}\text{gc}\mathbf{g}\text{cc}\mathbf{c} - 3'$	H – ras(wt)	target
$5' - \text{acacca}\mathbf{cc}\mathbf{t}\text{gctcca} - 3'$	N – ras(wt)	target

The level of transcription was measured by a semi-quantitative RT-PCR. Total mRNA extracted from untreated and PNA-treated cells was converted into cDNA and amplified by PCR, using primers specific for each *ras* gene. Moreover, the level of GAPDH transcription was chosen as a reference and measured in each sample. A typical result is reported in Figure 1.

It appears clear that in each sample, GAPDH is equally expressed, suggesting that the PNA treatment did not affect the expression of this housekeeping gene. Whereas the levels of H- and N-*ras* transcripts are found to be the same in untreated and PNA-treated cells, the K-*ras* transcripts are not detected in the cells treated with

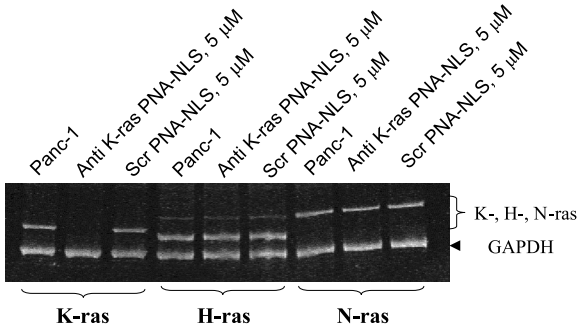

FIGURE 1 RT-PCR of mRNA extracts from untreated and 5 μM PNA-treated Panc-1 cells. Scr PNA-NLS: scramble PNAS conjugated to NLS; Anti PNA-NLS: N-tggagctgatggcgt-NLS.

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anti K-*ras* PNA-NLS. In contrast, K-*ras* transcripts are detected in untreated- and scramble-PNA-treated cells. Taken together, these data show that anti-K-*ras* PNA-NLS promotes a potent, gene-specific, antigene effect against K-*ras*. In keeping with previous observations,[4,7] this study suggests that the ability of NLS-conjugated PNA to specifically suppress transcription allows a wide range of applications in molecular therapy and gene functional studies.

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