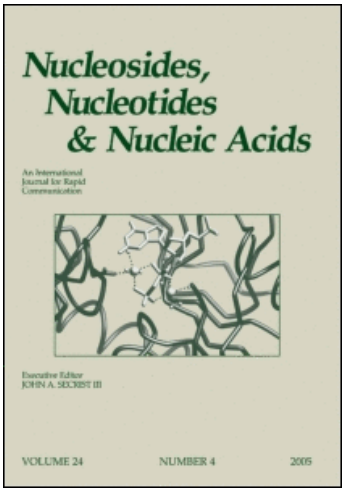

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Inhibition of Gene Expression by Peptide Nucleic Acids in Cultured Cells

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Inhibition of Gene Expression by Peptide Nucleic Acids in Cultured Cells

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ABSTRACT

We have tested in cultured cells the capacity of antisense and antigene PNAs to inhibit, in a sequence specific manner, the expression of oncogenes in leukaemia and pancreatic carcinoma cells. The results observed appeared promising and suggest that PNA may play in the future an important role in targeting disease-related genes.

Key Words: PNA; Cellular uptake; Antisense strategy; Antigene strategy.

Peptide nucleic acid (PNA) is a synthetic DNA analogue that is resistant to nucleases and proteases and binds with exceptional affinity to DNA and RNA.[1] Because of these properties PNA has the potential to become a powerful therapeutic agent to be used in vivo. Until now however, the use of PNA in vivo has not been thoroughly investigated. We have attempted to reduce the expression in cultured cells of the oncogenes *bcr/abl* and Ki-*ras* following two different strategies. The expression of *bcr/abl* in chronic myeloid leukemia KYO-1 cells was down-regulated using 13mer antisense PNAs (asPNA and parPNA), complementary to

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the b_2a_2 mRNA junction:

```
5′AGCGCAUUCCGCUGACCAUC AAUAAGGAAGAAG CCCUUCAGCGGCCAGUAGCA     b2a2
                  H2NOC-TTATTCCTTCTTC-NH2          asPNA
                    H2N-TTATTCCTTCTTC-CONH2        parPNA
                  H2NOC-CTTTTCTTCTCTA-NH2          scrPNA
```

The PNAs were linked with their *N*-terminus to fluorescein in order to perform uptake studies by confocal microscopy. In addition, PNA analogues with the *C*-terminus conjugated to the basic peptide PKKKRKV (NLS) were also synthesised, to enhance cellular uptake. We observed that not only NLS-conjugated but also free PNAs were taken up by KYO-1 cells through a receptor–independent mechanism.[2] In vitro asPNA, NLS-asPNA and, to a minor extent, parPNA inhibited reverse transcription at nM concentrations, suggesting that the binding between the b2a2 junction and PNA was very stable (using empirical equations we calculated that 10 μM asPNA:RNA should have a Tm of 52°C, while 10 μM parPNA:RNA should have a Tm of 37°C).[3,4] Using a semi-quantitative RT-PCR technique we found that the level of *bcr/abl* mRNA in KYO-1cells treated with 10 μM asPNA appeared to be reduced to less than 50% compared to control : cells untreated or cells treated with 10 μM scrPNA. This result would suggest the existence in the cell of a nuclease which recognises and degrades PNA:RNA duplexes. It is also possible that asPNA, which binds strongly to the mRNA target, impairs the reverse transcription reaction performed in the RT-PCR assay. Western blot analysis showed that the level of protein $p210^{BCR/ABL}$ was also reduced to 35% of the control, after the cells were exposed for 48 h to the PNA. Additionally, asPNA impaired cell growth by 50 ± 5% respect to the controls and inhibited the completion of the cell cycle. Surprisingly, the NLS-conjugated analogue of asPNA did not produced in KYO-1 cells the same biological effects as observed with asPNA.[2] This behaviour may be due to the fact that the positive charged peptide accumulates the PNA in the nucleus where the accessibility to the b2a2 target is more difficult than the accessibility of cytoplasmic b2a2.

PNA molecules were also used to inhibit specifically the expression of mutated Ki-*ras* in carcinoma Panc-1 cells, following in this case an anti-gene strategy. A 15mer PNA (anti GAT PNA) was designed with a sequence perfectly complementary to codon 12 of Ki-*ras* (GAT) located in the mutated allele (strong binding site). As this target is located in the nucleus, the PNA was conjugated to the NLS peptide. The expected PNA : DNA duplex should have a Tm of about 80°C.[3,4] However, as Panc-1 cells contain both mutated and wild type Ki-ras alleles, anti GAT PNA can also form a mismatched (C:A) PNA-DNA duplex with the wild type allele (codon GGT), with a predicted stability of about 60°C:

```
                          wild type allele      codon 12

...ATG ACTGAATATAAACTTGTGGTAGTTGGAGCT GGT GGCGTAGGG...
...tac tgacttatatttgaacaccatcaacctcga cca ccgcatccc...  Ki-ras
                              ::::::: ::: :::::
          anti GAT PNA      AcNH-TGGAGCT GAT GGCGT-PKKKRKV-CONH2
```


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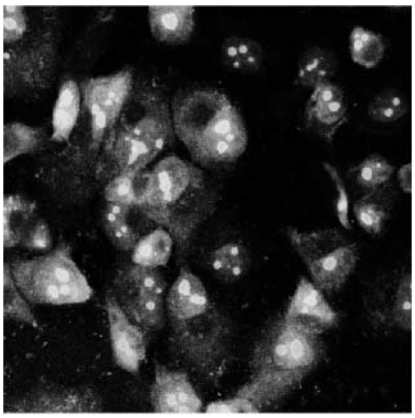

Figure 1. Confocal microscopy image of Panc-1 cells treated with fluorescinated anti GAT PNA.

mutated allele codon 12

```
...ATG ACTGAATATAAACTTGTGGTAGTTGGAGCT GAT GGCGTAGGG...
...tac tgacttatatttgaacaccatcaacctcga cta ccgcaTCCC..
                              ::::::: ::: :::::
        anti GAT PNA    AcNH-TGGAGCT GAT GGCGT-PKKKRKV-CONH2
```

As the hybridisation capacity of PNA is strongly influenced by mismatched bp, anti GAT PNA is expected to have a high affinity for mutated Ki-*ras* and a low affinity for the wild type Ki-ras. Thus, the designed antigene PNA should recognise only the target located in the mutated allele and not the parental sequence located in the wild type allele. Antigene anti GAT PNA was found to penetrate and accumulate in the nucleus of Panc-1 cells with high efficiency, due to its conjugation to the NLS peptide (Fig. 1). Moreover, anti GAT PNA significantly reduced both mRNA and protein levels. A single exposure to anti GAT PNA was sufficient to reduce, in a dose dependent manner, cell growth in Panc-1 cells. As expected, the effect of anti GAT PNA on the proliferation of BxPC3 cells, i.e., pancreatic carcinoma cells containing only wild type alleles and therefore weak sites, was much weaker. Br dU incorporation assays suggested that anti GAT PNA inhibits specifically the DNA synthesis in Panc-1 cells, indicating that the inhibition of Ki-ras has a profound effect on the cell cycle. These in vivo preliminary results indicated that PNA may be used to target only mutated alleles, without strongly interfering with the flow of genetic information involving the wild type allele.

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