Additive antisense effects of different PNAs on the in vitro translation of the PML/RARα gene

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ABSTRACT

The potential use of peptide nucleic acid (PNA) as a sequence-specific inhibitor of RNA translation is investigated in this report. Three different regions of the PML/RARα oncogene, including two AUG potential start codons, were studied as targets of translation inhibition by antisense PNA in a cell-free system. A PNA targeted to the second AUG start codon, which was shown previously to be able to suppress in vitro translation from that site completely, was used alone or in combination with another PNA directed to the first AUG, and a third PNA within the 5′-untranslated region (5′-UTR) of mRNA. When used alone, no PNA was able to completely block the synthesis of the PML/RARα protein. The 5′-UTR PNA was the most potent translation inhibitor when used as single agent. However, a near complete (≥90%) specific inhibition of the PML/RARα gene was obtained when the three PNAs were used in combination, thus obtaining an additive antisense effect.

INTRODUCTION

Antisense oligodeoxynucleotides (ODNs) have been developed to selectively block the expression of target genes (1). Phosphorothioates (2) represent, at present, the best characterized ODNs possessing increased biological stability; several phosphorothioate antisense drugs are undergoing clinical studies (3). Major obstacles to their use are represented by the reduced affinity for RNA when compared with unmodified ODNs, and by non-specific protein binding, which often leads to biological effects not related to their antisense activity (2,4). Attempts to improve ODN characteristics (specificity, nuclease resistance, pharmacokinetics, affinity for the target, cellular uptake, etc.) have led to the development of several derivatives, carrying methylphosphonate, methylmimino, phosphoramide, guanidine, 5-propynyl-pyrimidine, 2-O-allyl-ribose and many other, internucleotide linkages (5,6).

Peptide nucleic acid (PNA) is a DNA analogue in which the entire sugar–phosphate backbone is replaced by a polyamide (pseudopeptide) chain (7). PNA has been shown to bind single-and double-stranded DNA and RNA with high affinity (higher than DNA itself) and specificity (8). It is resistant to degradation in various cellular extracts and is able to block the activity of several DNA-acting enzymes and proteins, i.e. restriction endonucleases, transcription factors, methylases, DNA polymerases and telomerase (9–13). PNA has also been shown to inhibit transcription from double-stranded DNA (14,15). Finally, the translation of mRNA in cell-free systems is inhibited by duplex-forming PNAs directed to AUG regions or by triplex forming PNAs targeted to the coding region (15,16).

Abnormally activated proto-oncogenes are a common feature of tumor cells. They include membrane-bound as well as cytoplasmic and nuclear receptors, transcription factors, and molecules involved in signal transduction, cell-cycle progression or programmed cell death (17). Alterations in the expression and activity of such proteins determine uncontrolled cell proliferation in a complex, multistep process. Therefore, control of the expression of selected oncogenes represents a rational strategy in developing anti-cancer therapies. The PML/RARα fusion protein is responsible for >95% cases of acute promyelocytic leukemia (18) and is considered a model tumor-specific and transformation-related molecule. The PML/RARα gene contains two functionally active start codons, separated by 63 nucleotides (19). We showed previously that the in vitro translation of a PML/RARα mRNA containing only the second AUG is completely inhibited by a PNA oligomer that specifically recognizes that AUG region, with an activity ~40 times higher than a same-sequence standard ODN (15). In this report, we extend our analysis to the entire PML/RARα sequence, including the first AUG and the 5′-untranslated region (5′-UTR), which is important in controlling translation initiation. In fact, the length, nucleotide sequence and secondary structure of this region can influence both the choice of the initiator codon and the rate of translation (20). The effects of different PNAs on the in vitro translation of the entire PML/RARα fusion cDNA were studied. PNAs targeted to the first or second AUG were relatively inefficient in blocking translation, even when they were used together, whereas a PNA directed to the 5′-UTR of the gene was significantly more effective. However, the strongest inhibition was achieved when these three PNAs were combined, indicating that a complex
system, such as a complete gene, needs a multi-target antisense strategy to achieve complete translation inhibition.

**MATERIALS AND METHODS**

**PNAs and plasmids**

PNAs are designed to complement the PML/RARα mRNA at different positions. PNA#1 [H-GCAGGCTCCA TGGAC-Lys-NH₂] is directed against the PML/RARα first AUG start codon; PNA#2 [H-CATGGTGGCTCCTG-Lys-NH₂] is targeted towards the second AUG; and PNA#3 [H-AGATCTGGAGTGCG-Lys-NH₂] hybridizes with a sequence in the 5′-UTR of the transcript. Positions of PNAs relative to the PML/RARα mRNA are depicted in Figure 1. All PNAs were obtained by solid-phase synthesis as described (21).

Plasmid BCR1 contains PML/RARα cDNA starting from the second AUG start codon region cloned in the pGEM3 vector (15). Plasmid P/R, carrying the complete PML/RARα cDNA inserted in the pSG5 vector, was a gift of Dr F. Grignani (University of Perugia). Plasmid pGF-1 encodes the PTC-2 fusion protein (22). All sequences are under the transcriptional control of the T7 promoter.

**In vitro translation**

The PML/RARα protein was synthesized from the P/R or BCR1 plasmid using the TNT T7-coupled reticulocyte lysate system (Promega). Briefly, DNA (1 µg), TNT buffer, amino acids, RNase inhibitor (20 U), l-[35S]methionine (20 µCi), T7 RNA polymerase and rabbit reticulocyte lysate were mixed in the presence of the desired concentrations of PNAs and incubated for 90 min at 30°C. Samples were then boiled for 3 min in loading buffer (60 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, bromophenol blue), loaded on a 7.5% SDS–polyacrylamide gel, and proteins detected by autoradiography. The PTC-2 protein was always synthesized in the same tube as an internal control of specificity. The ratios between P/R and BCR1 plasmids and the pGF-1 control plasmid were 8:1 and 2:1, respectively. The intensity of protein bands on the films was determined by scanning on the 620 CCD densitometer (Bio-Rad) and the data analyzed by the 1-D Analyst II software (Bio-Rad).

**RESULTS**

As we have shown previously (15), a PNA complementary to the second ATG of the PML/RARα gene (PNA#2) can specifically block the in vitro translation of a PML/RARα mRNA that starts from the second AUG (Fig. 2). An additional in-frame start codon is present 63 bases upstream in the complete PML/RARα transcript (19); when a plasmid containing the complete cDNA sequence was translated in vitro, PNA#2 proved inefficient when used alone (Fig. 3 A). The apparent difference in the amount of PML/RARα protein synthesized in the two experiments is mostly due to a lower ratio between PML/RARα and the control plasmid in Figure 2 (see also Materials and Methods). Another PNA (#1) was therefore designed to hybridize to the first AUG, and was used alone (Fig. 3 D, lanes 2–4), or in combination with PNA#2 (Fig. 3 B); only a partial inhibition of PML/RARα synthesis was obtained, without a complete block. Another PNA targeted to the first AUG and partially overlapping the PNA#1 target sequence, was completely ineffective, and was not analyzed further (not shown). These data show that PNAs against the two AUG codons were not sufficient to achieve a complete in vitro translation inhibition of PML/RARα when the 5′-non-coding region of the gene was present.

A third PNA (#3) was then synthesized in order to bind in the 5′-UTR of the messenger. This PNA binds 30 bp upstream of the first AUG, and was used to evaluate the relevance of this region for translation initiation. As shown in Figure 3C, PNA#3, when used as a single agent, failed to produce complete inhibition in the nanomolar range, although it showed a good inhibitory effect at 2 µM. Thus, while PNA#3 did not produce a complete block, it resulted in superior antisense effects compared with PNA#1 and #2.

We then investigated the effect of combining these three PNAs (#1, #2 and #3), to see whether it was possible to increase their
Figure 3. Effects of the three PNAs on the translation of the entire PML/RARα gene. P/R (full-length) plasmid was used instead of BCR1, with PNA#2 alone (A), PNA#1 and #2 (B) or PNA#3 alone (C). (D) Representative of four experiments, the inhibition obtained by PNA#1 alone is compared with that given by the same concentrations of PNA#1 in the presence of PNA#2 and #3. Concentrations of the PNAs are indicated above the figure.

Figure 4. (A–D) Densitometric analysis of data presented in Figure 3A–D, respectively. The intensity of the bands was calculated as peak area. PML/RARα protein levels were normalized over the control protein synthesis. The data reported represent the mean ± SD of three [or four in (D)] independent experiments.
antisense effect. Figure 3D shows the results of this combination: lanes 2–4 represent the results produced by PNA#1, while lanes 5–7 represent the combination of PNA#1, #2 (used at 0.6 µM as in Fig. 3B) and #3 at 0.8 µM. Translation inhibition of 90% to almost 100% was obtained by the simultaneous presence of the three PNAs at concentrations at which they failed to produce a significant (>50%) inhibition when used as single agents. The level of the PTC2 protein, co-translated in the same reaction as an internal control, was not affected by the anti-PML PNAs. Therefore, the inhibition obtained can be considered specific. Figure 4 shows the results from three different experiments (of which Fig. 3 reports one representative example) evaluated by densitometric analysis. As shown in panels A and B, PNA#1 and #2 are not able to achieve complete inhibition, while PNA#3 (panel C) is more active, but at concentrations >1 µM. In Figure 4D, the combination of the three oligomers is presented and compared with the use of PNA#1 alone (as shown in Fig. 3D).

PNA#1–3 failed to bind dsDNA and did not inhibit the in vitro transcription of PML/RARα plasmids, as can be expected from mixed-sequence PNAs (not shown).

We can conclude from these data that the complete translation inhibition of the PML/RARα gene required the blocking of both of the two AUG sites and the untranscribed region situated 5’ of the first AUG codon.

To assess the possibility that the three PNAs produce a co-operative antisense effect, we checked the extent to which one PNA could modify the IC50 (defined as the PNA concentration at which 50% translation inhibition occurs) of a second PNA. Table 1 reports two representative experiments: PNA#1 has an IC50 of 1.07 µM, while the IC50 of PNA#3 is 0.38 µM. When used combined at their IC50, one should expect ~25% expression of the control PML/RARα protein (50% of 50%) in the case of an additive effect, while a co-operative effect should produce an higher inhibition. As shown in Table 1, two experiments produced 21 and 30% inhibition respectively, with a mean ± SD of 25.5 ± 6.4. Therefore, PNA#1 and #3 produced an additive effect, yet without showing significant co-operation.

DISCUSSION

PNA/RNA duplexes are not substrates for RNase H (16), and antisense activity of PNAs must rely on different mechanisms, such as, for example, steric blocking of scanning, assembling or elongating ribosomes. Translation elongation arrest appears to require a (PNA)2–RNA triplex and thus needs a homopurine target of 10–15 bases; mixed sequence duplex-forming PNAs have so far been shown to exhibit antisense activity only when targeted to the AUG region. These requirements limit the possible target sequences in a gene. In addition to the characteristics of the antisense molecule, the properties of the targeted sequence must also be considered. When targeting a real gene such as an oncogene, one has to take into account various difficulties that are not present in model systems, such as the presence of multiple translation start sites (19) that may lead to the synthesis of functional isoforms or the formation of secondary structures, or protein binding that may render some mRNA sites inaccessible.

In this report, we present the effects of three types of antisense PNAs on the in vitro expression of the PML/RARα gene: one PNA was complementary to the first AUG (initiation site) and the second bound to a sequence in the coding region that included the second AUG, from which a biologically active protein can be synthesized (23). The third PNA was directed to a site in the 5’-UTR of the mRNA, where the translation machinery is considered to assemble; in fact, it is known that eukaryotic ribosomes select the translation start codon by scanning the mRNA sequence from the 5’-cap until they reach an AUG triplet surrounded by an appropriate consensus sequence (20). During this process, the ribosomal machinery is not entirely assembled (the large subunit joins the complex after the recognition of the start AUG), and probably not firmly associated with the mRNA; thus, the translation complex could be more sensitive to a block, or the RNA could be more accessible to PNA in this region. Indeed, of the three PNAs tested, PNA#3 (the one directed against this region) achieved the highest (although incomplete) inhibition of translation, when used alone.

The three PNAs used in this study showed limited effects when tested separately, but they provided an efficient inhibition when used together. In fact, at concentrations between 0.6 and 0.8 µM (at which all PNAs failed to reach relevant blocking when used singularly), almost total inhibition was obtained by the combined use of the three PNAs. The combined effect was shown to be additive rather than co-operative; thus, it is conceivable that each PNA acted on its own target sequence, contributing a fraction of the total inhibition, without interfering with the other PNAs. These results indicate that the PNA targeting of complex RNAs such as PML/RARα requires the effective blocking of different target sequences on the 5’ part of the messenger.

CONCLUSION

These data show that a 5’-UTR PNA target may be significantly more potent than an AUG target and that an effective translation inhibition can be achieved by combining PNA targeted at 5’-UTR and AUG regions.

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