Modified peptide nucleic acids are internalized in mouse macrophages RAW 264.7 and inhibit inducible nitric oxide synthase

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Abstract Overexpression of inducible nitric oxide synthase causes the production of high levels of nitric oxide, which, under pathological conditions, leads to immunosuppression and tissue damage. The results recently obtained using peptide nucleic acids, rather than traditional oligonucleotides as antigen and antisense molecules, prompted us to test their efficacy in the regulation of nitric oxide production, thereby overcoming the obstacle of cellular internalization. The cellular permeability of four inducible nitric oxide synthase antisense peptide nucleic acids of different lengths was evaluated. These peptide nucleic acids were covalently linked to a hydrophobic peptide moiety to increase internalization and to a tyrosine to allow selective ^{125}I radiolabelling. Internalization experiments showed a 3–25-fold increase in the membrane permeability of the modified peptide nucleic acids with respect to controls. inducible nitric oxide synthase inhibition experiments on intact stimulated macrophages RAW 264.7 after passive permeation of the two antisense peptide nucleic acids 3 and 4 demonstrated a significant decrease (43–44%) in protein enzymatic activity with respect to the controls. These data offer a basis for developing a good alternative to conventional drugs directed against inducible nitric oxide synthase overexpression.

Key words: Peptide nucleic acid; Inducible nitric oxide synthase; RAW 267.4; Internalization

1. Introduction

Nitric oxide (NO) is an important vascular and neuronal messenger and a cytotoxic agent produced by three different isoforms of NO synthase, eNOS, nNOS and inducible NOS (iNOS) [1–3]. The first two forms are constitutively expressed while iNOS was first described in macrophages and is expressed as a consequence of various inflammatory stimuli [4]. Under these circumstances, NO mediates anti-microbial and anti-tumoral activities of the immune system, but long-term NO overproduction causes tissue damage and other serious diseases [1–4]. These two opposite effects imply that the organism must regulate NO synthesis precisely. Pharmacological research has developed many synthetic inhibitors able to modulate the production of NO in pathological conditions [4]. The main problems concern the aspecific activity of the enzymatic inhibitors that can negatively interact with all three isoforms of NOS and often interfere with other unrelated metabolic cellular pathways [2]. Thus, selective blockade of iNOS activity is a major goal and the use of an antisense strategy to achieve this result would overcome the undesired side-effects of conventional inhibitors.

Recently, various studies have demonstrated that specific oligonucleotide analogs are able to permeate cell membranes through endocytosis and to some degree to inhibit iNOS [5,6]. In the last years, the partial inhibition of iNOS using a specific antisense oligonucleotide in an 'in vivo' shock model has also been described [7].

In this study, a different approach to the antisense strategy was used, by means of experiments involving internalization and inhibition of translation by a novel class of oligonucleotide analogs named peptide nucleic acids (PNA). In these molecules, the entire deoxyribose phosphate moiety is replaced by an uncharged pseudo-peptide backbone consisting of *N*-(2-aminoethyl)glycine units linked to the purine and pyrimidine bases of natural nucleic acids [8]. These structures form very stable complexes with complementary DNA or RNA and are resistant to enzymatic degradation in biological fluids and tissues [8–12]. We have previously described the synthesis and characterization of a specific PNA able to inhibit the translation of iNOS in a cell-free model [13]. In the present study, we have addressed the design and synthesis of modified iNOS-directed antisense PNA molecules to be used in intact cells and modified accordingly. Stimulated RAW 264.7 murine macrophages were investigated with respect to their susceptibility to these PNA molecules and to other PNAs designed as negative controls.

2. Materials and methods

2.1. PNA designing and synthesis

Three antisense PNAs of different lengths (Table 1), 5 (PNA 1), 10 (PNA 2) and 14 (PNA 3) bases, respectively, complementary to the homopurinic regions 238–242, 238–247, 238–251 of the iNOS mouse cDNA (accession number M84373), were synthesized and chemically modified by the introduction of a hydrophobic peptide moiety at the carboxy-terminal to increase the membrane permeability [14] and of a tyrosine at the amino-terminal to allow selective radiolabelling with ^{125}I.

Three control molecules lacking the hydrophobic moiety (PNA 1a, 2a, 3a) were synthesized as well (Table 1). A scrambled PNA (PNA 3c) of 14 bases with the hydrophobic portion was obtained and used as negative control to evaluate the selective antisense activity of PNA 3 in intact cell cultures. Two other PNAs, the first with an antisense sequence identical to PNA 3 (PNA 4) and the second with a

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Abbreviations: Boc, tert-butoxycarbonyl; ESMS, electrospray mass spectrometry; iNOS, inducible nitric oxice synthase; LPS, lipopolysaccharide; MBHA-PS, 4-methyl-benzhydril-amine-polystyrene; PNA, peptide nucleic acid; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid

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scrambled sequence identical to PNA 3c (PNA 4c) (Table 1), as negative control, were synthesized with a hydrophobic peptide moiety of four rather than of six amino acids. The PNA-chimera (1, 2, 3, 3c, 4, 4c) and the control molecules (1a, 2a, 3a) were manually synthesized using the standard method of solid phase peptide synthesis which follows the tert-butoxycarbonyl (Boc) strategy [15,16] with minor modifications [14]. Briefly, 25 μmol of 4-methyl-benzhydril-amine-polystyrene (MBHA-PS)-Phe-Boc deprotected resin (Novabiochem AG, Laufelfingen, Switzerland) in the case of PNA-chimera, or alternatively, of MBHA-PS-Gly-Boc deprotected resin for control PNAs, was treated for 20 min at 40°C with a coupling reaction mixture containing five equivalents (eq) of the appropriate Boc-amino acid (Novabiochem) or Boc-PNA monomer (Perseptive Biosystems, PRIMM, Milan, Italy), 4.5 eq of *o*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (PRIMM), five eq of *N,N*-diisopropilethylamine (Fluka Chemie AG, Buchs, Switzerland), 7.5 eq of Sim-Collidine (Fluka) at 0.1 M monomer (or amino acid) final concentration in anhydrous *N*-methyl pyrrolidone (Fluka). All the synthesized compounds were purified by reverse phase high performance liquid chromatography (RP-HPLC) and their molecular weights finally confirmed by electrospray mass spectrometry (ESMS) (see Section 2.6 below).

2.2. Iodination of PNAs

PNAs 1, 1a, 2, 2a, 3, 3a and 4 were radioactively labelled with ^{125}I on the amino-terminal tyrosine following the method described in [17]. All PNAs employed in the following experiments had a specific activity of 0.35 μCi/nmol.

2.3. Cell cultures

All experiments were performed on the mouse macrophage cell line RAW 264.7 (obtained from the American Type Culture Collection Rockville, MD, USA) cultured in DMEM containing 4 mM glutamine supplemented with 10% FCS [13]. Cell stimulation to promote iNOS synthesis in intact murine macrophages was achieved by adding 1 ng/ml lipopolysaccharide (LPS) (Sigma, Milan, Italy) to the cell cultures 18 h before processing cells to obtain lysates in which the iNOS enzymatic activity was evaluated.

2.4. Internalization and metabolism of PNAs in RAW 264.7 cells

PNAs 1, 1a, 2, 2a, 3, 3a and 4, labelled with ^{125}I and with a specific activity of 0.35 μCi/nmol, were incubated at a final concentration of 1 μM for each PNA with a RAW 264.7 cell culture. Briefly, exponentially growing cells (10^6/ml) were treated with each PNA for 6, 12, 24 h (or 12, 24, 78 h with PNA 4) at 37°C and 6% CO_2. After incubation, cells were extensively washed with ice-cold PBS, resuspended in 300 mM sucrose and 10 mM Tris-HCl, pH 6.5, (lysis buffer) at a dilution of 2.5×10^6 cells/ml and sonicated to disrupt cell membranes. Aliquots were withdrawn to evaluate the total cellular radioactivity in a Packard γ-counter, then, samples were ultracentrifuged on a TL-100 Beckman for 15 min at $100\,000\times g$, the membrane pellet was resuspended in lysis buffer (the same volume as the supernatant) and the radioactivity was measured in the γ-counter. In addition, incubations of 24 h with 1 mM PNA 4 were carried out at 37°C using a RAW 264.7 cell lysate (obtained by five cycles of freezing at −80°C for 30 min and thawing at 37°C for 10 min). In a parallel incubation, intact cultured RAW 264.7 (10^6 cell/ml) were treated for 72 h with 1 μM PNA 4 (^{125}I specific activity of 0.35 μCi/nmol). At 2, 6, 24 h, aliquots of the lysate, or after 72 h, samples of intact cells, were withdrawn (the cells washed with ice-cold PBS) and extracted with one volume of methanol added to lysate or intact cells resuspended in PBS.

PNA 4 and its metabolites in lysate extracts were analyzed by RP-HPLC directly coupled to ESMS as described previously [14]. The metabolites of the same compound present in the cell culture extracts were separated by RP-HPLC (see Section 2.6) and during analysis, fractions of 0.5 ml were collected for radioactivity evaluation in a γ-counter. Identification of metabolites in the cell culture incubation was performed by comparing the retention time of each HPLC peak with HPLC-ESMS patterns of the lysates in which each metabolite peak occurring in the HPLC chromatogram was assigned a mass via ESMS analysis directly coupled to the HPLC.

2.5. NOS assay

The protein concentration was analyzed using the standard method of Bradford [18]. The NOS assay was performed by evaluating the conversion of [^{3}H]arginine to [^{3}H]citrulline in cell lysates. Exponentially growing RAW 264.7 cells were cultured in 25 cm^2 flasks for 4 days with 1 μM PNA 3 or 4 and its corresponding controls, PNA 3c or 4c, or alternatively for 7 days with 1 μM PNA 4 and 4c. In all cases, stimulation of RAW 264.7 cells with 1 ng/ml LPS was performed 18 h before processing cells to obtain the cytosolic fraction. Briefly, cells were collected and extensively washed with ice-cold PBS, then, resuspended in 300 μl 15 mM HEPES, pH 7.5 and lysed by freezing-thawing (see Section 2.4). An enzymatic assay of cytosolic glucose-6-phosphate-dehydrogenase was performed on each sample to confirm the disruption of cell membranes and showing similar activities in treated and untreated cells (data not shown). The lysates were then centrifuged at $100\,000\times g$ for 20 min, the cytosolic fractions collected and 200 μl aliquots were incubated for 1 h at 37°C with 5 mM EDTA, 0.5 mM NADPH, 60 μM BH_4, 1 mM dithiothreitol, 1 mM $MgCl_2$ and 0.1 mM L-arginine, 2 mCi [2,3,4-^{3}H]L-arginine monohydrochloride (45–70 Ci/mmol, NEN Life Science Products, Boston, MA, USA) [19]. Lysate proteins were then precipitated by addition of 5.5% (final concentration) TCA to the incubation mixture. Residual TCA was removed by a triple extraction with 20 volumes of diethyl ether as described [20], samples were diluted with one volume of 15 mM HEPES, pH 7.5, and finally, 100 μl aliquots were analyzed by HPLC.

2.6. Analyses and characterization of products and cellular metabolites

All synthesized compounds were analyzed and purified by RP-HPLC. The analysis of the crude synthesis products was performed using an HP 1090 HPLC equipped with a Waters C_{18} μBondapack column (3.9×300 mm), while the purification of individual compounds was obtained on a Shimadzu LC-9A preparative HPLC equipped with a Waters C18 μBondapack column (19×300 mm). The solvent program for both analyses was a gradient starting with 100% solvent A for 5 min, linearly increasing to 60% solvent B in 30 min and up to 100% B in 5 min. Solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in acetonitrile. The diode array detector was set at 260 nm during the analyses. Mass spectra of each compound were acquired using a single quadrupole HP Engine 5989-A equipped with an electrospray ion source (ESMS) and set in the positive ion mode and confirmed using a Kompact MALDI 4 TOF spectrometer (Kratos Analytical, Manchester, UK). HPLC analyses of the lysate extracts obtained from the NOS assay incubation mixtures were performed on a reverse phase HP ODS Hypersil column (100×4.6 mm, 5 μm particulate size) at a flow rate of 0.5 ml/min. Analyses were performed by applying a linear gradient starting with 100% A and linearly increasing to 100% B in 20 min, buffer A being 25 mM sodium acetate and 15 mM sodium heptanesulphonate, pH 4.35, and buffer B the same as A with the addition of 30% methanol. This analysis allowed a good peak resolution of arginine, citrulline and ornithine as described [21]. 0.5 ml aliquots of eluate were collected and the radioactivity was counted in a Packard liquid scintillator.

3. Results

3.1. Synthesis and characterization of PNAs

Synthesis of the different PNA sequences had final yields ranging between 50% and 60% with a purity of 95–98% after preparative RP-HPLC purification of products. In all cases, the mass spectra of the purified PNAs showed $[M+H]^+$ ions consistent with the molecular weights of the expected molecules.

3.2. Internalization and metabolism in RAW 264.7

All PNAs with different length antisense sequences targeted to iNOS mRNA, with or without the hydrophobic peptide moiety (PNAs 1, 1a, 2, 2a, 3, 3a, 4), were first tested for their efficacy on intact RAW 264.7 cells. In these experiments, the roles of the lipophilic peptide vehicle and of its different chain lengths as well as the positive or negative contribution to the membrane permeability of different size PNA sequences were compared in time course experiments. After incubations of the

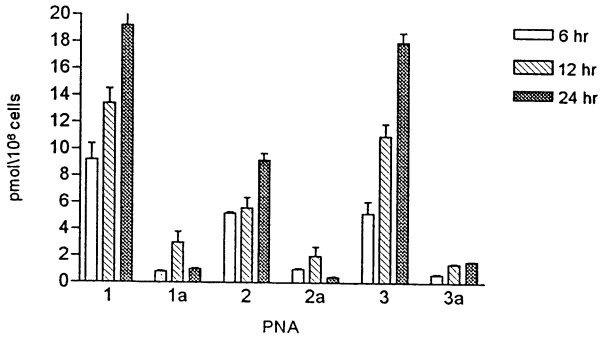

Fig. 1. Cytosolic levels of different PNA molecules with or without the hydrophobic peptide vehicle. Data, expressed as pmol/10^6 cells, are taken from the experiment indicated in Table 2. Indicated S.E.M. are based on triplicate experiments.

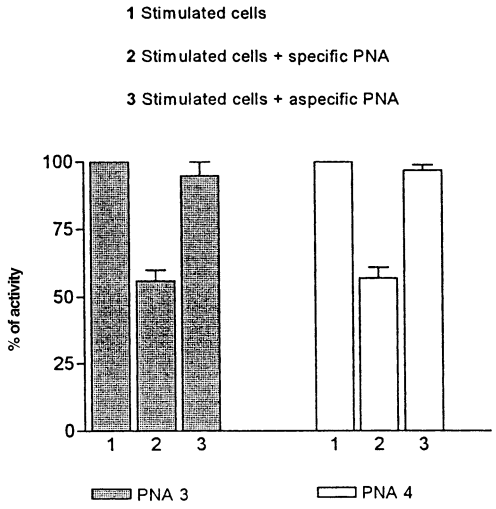

Fig. 2. Inhibition of iNOS activity in RAW 264.7 cells by PNA 3 (gray columns) after 4 days of incubation and by PNA 4 (white columns) after 7 days. PNA 3 shows a 44% inhibition (second gray column), while PNA 4 shows a 43% inhibition (second white column) compared to controls. S.D. derive from triplicate experiments.

iodinated PNAs, the radioactivity in intact cells and in membrane and cytosolic fractions was evaluated as reported in Table 2 and Fig. 1. Modified PNAs 1, 2, 3 and 4 showed much higher internalization values with respect to control PNAs 1a, 2a and 3a at all incubation times. Specifically, the rates of internalization, expressed as pmol/10^6 cells, for control PNAs 1a, 2a and 3a were found to be 3–25 times lower with respect to modified PNAs 1, 2, 3 and 4 in intact cells (see the 'T' column of Table 2). Analysis of the membrane and cytosolic fractions, indicated by the 'M' and 'C' columns, respectively, of Table 2, revealed an asymmetric distribution of the modified PNA molecules in cells, while similar values were found for unmodified PNAs in the same fractions, as predicted from the chemical structures of the synthesized molecules. In particular, the experiments revealed that 5–20% of the modified PNAs were in the cytosolic fraction and thus available for iNOS mRNA complementation and inhibition. Furthermore, this concentration in the cell cytosol was significantly higher with respect to the controls, as reported in Fig. 1 (see the 'C' column in Table 2) that displays the great difference in cytoplasmic internalization of the modified PNAs 1, 2, 3 upon PNAs 1a, 2a, 3a. Lower values were obtained for PNA 4, with a maximum rate of internalization in intact cells after 72 h of 62.5 pmol/10^6 cells and of 7.7 pmol/10^6 cells only in the cytoplasm.

All these data confirm the considerable advantage obtained upon introducing a hydrophobic peptide vehicle covalently linked to the PNAs over the control molecules. Confirmation of the intracellular stability of the PNA moiety itself and of the tyrosine linkage positioned at the amino-terminal end was needed to validate the internalization studies based on the stable ^{125}I labelling of the molecules. This requirement prompted us to investigate the metabolism of iodinated PNA 4 in intact RAW 264.7 cells after 72 h of incubation at a 1 μM PNA concentration in culture medium. The metabolic pattern of conversion of PNA 4 occurring in intact cells after a 3 day incubation showed, as already described in [14], a stepwise removal of each amino acid of the peptide moiety, probably due to carboxypeptidase activities, generating the following percentages of molecular species: 7.6% intact PNA 4 (M) = 4524 (M.W.), 17.8% (M-(Gly-Phe)) = 4322, 23.6% (M-(Gly-Phe-Leu)) = 4209 and 50.9% (M-(Gly-Phe-Leu-Phe)) = 4062. No evidence of enzymatic cleavage of the ^{125}Ilabelled tyrosine residue covalently linked to the amino-terminal end and of the PNA moiety itself was observed. This stability strongly validates the data of cellular uptake of all PNAs synthesized and tested in this study and expressed as ^{125}I radioactivity incorporated by intact cells and subsequently in membrane and cytosolic cellular fractions.

3.3. Inhibition of iNOS in RAW 264.7 lysates

RAW 264.7 were incubated for 4 days with PNAs 3, 3c, 4 and 4c and for 7 days with PNA 4 and 4c and stimulated. NOS activity was then evaluated in the cytosolic fraction. To preserve the cellular growth and viability, the above-mentioned prolonged incubations were performed in DMEM sup-

Table 1
Nucleotide sequences of the synthesized PNA molecules

PNA	Sequence	bp
PNA 1	3′-CONH-Phe-Leu-Phe-Leu-Phe-Leu-CTTTC-Lys-Tyr-NH_2-5′	238–242
PNA 1a	3′-$CONH_2$-Gly-CTTTC-Lys-Tyr-NH_2-5′	238–242
PNA 2	3′-$CONH_2$-Phe-Leu-Phe-Leu-Phe-Leu-CTTTCTCCTT-Lys-Tyr-NH_2-5′	238–247
PNA 2a	3′-$CONH_2$-Gly-CTTTCTCCTT-Lys-Tyr-NH_2-5′	238–247
PNA 3	3′-$CONH_2$-Phe-Leu-Phe-Leu-Phe-Leu-CTTTCTCCTTTTCC-Lys-Tyr-NH_2-5′	238–251
PNA 3a	3′-$CONH_2$-Gly-CTTTCTCCTTTTCC-Lys-Tyr-NH_2-5′	238–251
PNA 3c	3′-$CONH_2$-Phe-Leu-Phe-Leu-Phe-Leu-CTTTTTCCCTCTTC-Lys-Tyr-NH_2-5′	–
PNA 4	3′-$CONH_2$-Gly-Phe-Leu-Phe-Leu-CTTTCTCCTTTTCC-Lys-Tyr-NH_2-5′	238–251
PNA 4c	3′-$CONH_2$-Phe-Leu-Phe-Leu-CTTTTTCCCTCTTC-Lys-Tyr-NH_2-5′	–

Nucleotidic sequences of the synthesized antisense and scrambled PNA molecules complementary to the 5′ homopurinic encoding region of iNOS mouse mRNA.

Table 2
PNA internalization values (pmol/10^6 cells)

Time	6 h			12 h			24 h			72 h		
PNA	T	M	C	T	M	C	T	M	C	T	M	C
PNA 4	–	–	–	22.4	19.9	2.4	17.0	14.7	2.2	62.5	54.7	7.7
PNA 3	87.2	82.0	5.2	74.0	63.0	11.0	98.0	80.0	18.0	–	–	–
PNA 3a	2.8	2.2	0.6	7.2	5.8	1.4	11.6	10.0	1.6	–	–	–
PNA 2	41.2	36.0	5.2	68.2	62.6	5.6	153.4	144.0	9.4	–	–	–
PNA 2a	5.0	4.0	1.0	5.4	3.4	2.0	1.2	0.8	0.4	–	–	–
PNA 1	193.2	184.0	9.2	76.6	63.2	13.4	147.2	147.2	19.2	–	–	–
PNA 1a	7.8	7.0	0.8	25.0	22.0	3.0	7.6	7.6	1.0	–	–	–

PNA internalization values expressed as pmol/10^6 cells at different incubation times in a RAW 264.7 cell culture. The 'T' column displays the total cellular internalization values while the 'M' and 'C' columns show the PNA partition between the lipid (i.e. membrane) and cytosolic fractions of total cellular values, respectively.

plemented with 10% FCS. With reference to this, no significant interactions between PNA and serum proteins have been reported [12,22], strongly suggesting that all PNA dissolved in the medium was always available for cellular internalization.

After 4 days of incubation with PNA 3 (Fig. 2, gray columns), stimulated cells showed a decrease of 44% of iNOS activity (second gray column), while PNA 3c did not affect it at all (third gray column). PNA 4 and PNA 4c showed no significant inhibition after 4 days of incubation (data not shown), while a prolonged incubation for a total period of 7 days again showed (Fig. 2, white columns) a selective inhibition of iNOS by PNA 4 corresponding to a 43% decrease in enzymatic activity with respect to control stimulated cells (second white column) and to PNA 4c (third white column).

4. Discussion

Inducible NOS is the enzyme responsible for the production of relatively large amounts of NO in macrophages in response to LPS and cytokine stimulation in general potentiating the host's immune defense system [4]. However, a pathological upregulation of the NO synthesis leads to systemic detrimental effects and self-destruction since NO cannot be stored or inactivated by conventional mechanisms after release [1–4]. Therefore, a variety of drugs interfering with the activity of NOS have been developed but they are often toxic and have a low specificity as they block other enzymes as well [2].

This background prompted us to synthesize, in a previous work [13], a specific anti-NOS PNA for the 'in vitro' evaluation of the sequence-selective inhibition of iNOS mRNA translation, which suggested the possibility of developing an alternative pharmacological control on NO overproduction, based upon an antisense PNA strategy. The homopyrimidinic PNA sequence proved to be a potent 'in vitro' selective inhibitor of iNOS synthesis by blocking its mRNA translation, so, we were encouraged to test the specific antisense effect of this molecule on intact stimulated macrophages. However, to this purpose, we had to tackle the obstacle of crossing intact cellular membranes, a problem common to oligonucleotides and their analogs and to PNAs as well [23,24]. The internalization study performed on four different length PNAs (Table 2 and Fig. 1), covalently linked to a hydrophobic peptide, showed that the cellular internalization rate was significantly higher (3–25 times) with respect to control PNAs lacking the hydrophobic moiety (Table 2). Furthermore, this study revealed that the linkage to a longer lipophilic peptide (PNA 1, 2, 3) results in a much more rapid internalization with respect to the shorter hydrophobic moiety (PNA 4).

All these data suggest a simple way of modifying PNAs in order to achieve high intracellular concentrations of these molecules. That this condition is an important prerequisite has been underlined in a recent work [23] where a comparison of the cellular uptake of different oligonucleotide analogs was performed, showing lower internalization rates for unmodified PNAs with respect to other antisense molecules such as phosphorothioates and alkylamino-phosphodiesters. In our study, similar, and sometimes higher, cellular uptakes were obtained, compared to the other oligonucleotide analogs. As a follow-up, the ability of PNAs 3 and 4 (Fig. 2) to exert a specific inhibitory effect on iNOS expression within the cytoplasmic compartment of intact RAW 264.7 cells was evaluated. The selective antisense effect of the two PNAs was evaluated by measuring the extent of inhibition of conversion of [^{3}H]arginine to [^{3}H]citrulline by iNOS in stimulated cell lysates (Fig. 2). These experimental conditions excluded calcium-dependent activity (e.g. constitutive NOS isoforms) and re-shuttling of citrulline to arginine by arginine-succinate synthase (data not shown), an event that has been recently described in intact RAW 264.7 cells [25]. The HPLC analysis allowed for the separation of radioactive species (arginine, citrulline and ornithine) generated during incubations of the lysates, performing a much more precise assay compared with well-known conventional methods based upon cationic exchange columns [21]. In this respect, the presence of LPS-induced arginase activity in macrophages, with the consequent production of variable amounts of ornithine that may alter the background of cationic exchange columns, has recently been described [26].

The results shown in Fig. 2 indicate a 44% iNOS-specific inhibition for PNA 3 after 4 days of incubation and a 43% iNOS inhibition for PNA 4 after 7 days of incubation. The different times required to obtain the maximum antisense effect of the two molecules are explained by the different length of hydrophobic moieties linked to PNAs 3 and 4 that confer to the first molecule a more rapid internalization and, subsequently, inhibitory ability.

These encouraging results offer a promising new alternative to conventional drugs directed against macrophagic iNOS overexpression but also to oligonucleotides [6] and their phosphorothioate analogs [5] already tested for their specific antisense effect on this enzyme's activity. Furthermore, an in vivo study of septic shock on a rat model [7] also reveals the advantages of an antisense therapy against iNOS macrophagic

overexpression, showing highly promising results in this area and indicating the possibility of developing a PNA therapy on an 'in vivo' rat model. This goal could be achieved in several ways: by introducing suitable modifications enhancing internalization of antisense PNA, by using antigene PNA linked to nuclear localization signals or by eventually targeting PNA specifically to the macrophagic compartment through the coupling to macrophage-membrane permeable signal peptides. These modifications may in principle generate an ideal drug, able to selectively exert its highly specific pharmacological action at the proper site of the protein malfunction, thereby reducing side-effects and systemic toxicity.

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