Antigene peptide nucleic acid specifically inhibits MYCN expression in human neuroblastoma cells leading to cell growth inhibition and apoptosis

Roberto Tonelli, Stefania Purgato, Consuelo Camerin, Raffaele Fronza, Fabrizio Bologna, Simone Alboresi, Monica Franzoni, Roberto Corradini, Stefano Sforza, Andrea Faccini, Jason M. Shohet, Rosangela Marchelli, and Andrea Pession

1Department of Pediatrics, University of Bologna, Sant’Orsola-Malpighi Hospital, Bologna, Italy; 2Department of Organic and Industrial Chemistry, University of Parma, Parma, Italy; and 3Center for Cell and Gene Therapy, Texas Children’s Cancer Center, Baylor College of Medicine, Houston, Texas

Abstract
We developed an antigene peptide nucleic acid (PNA) for selective inhibition of MYCN transcription in neuroblastoma cells, targeted against a unique sequence in the antisense DNA strand of exon 2 of MYCN and linked at its 3′ terminus to a nuclear localization signal peptide. Fluorescence microscopy showed specific nuclear delivery of the PNA in six human neuroblastoma cell lines: GI-LI-N and IMR-32 (MYCN-amplified/overexpressed); SJ-N-KP and NB-100 (MYCN-unamplified/low-expressed); and GI-CA-N and GI-ME-N (MYCN-unamplified/unexpressed). Antiproliferative effects were observable at 24 hours (GI-LI-N, 60%; IMR-32, 70%) and peaked at 72 hours (GI-LI-N, 80%; IMR-32, 90%; SK-N-KP, 60%; NB-100, 50%); no reduction was recorded for GI-CA-N and GI-ME-N (controls). In MYCN-amplified/overexpressed IMR-32 cells and MYCN-unamplified/low-expressed SJ-N-KP cells, inhibition was recorded of MYCN mRNA (by real-time PCR) and N-Myc (Western blotting); these inhibitory effects increased over 3 days after single treatment in IMR-32. Antigene PNA induced G1-phase accumulation (39–53%) in IMR-32 and apoptosis (56% annexin V–positive cells at 24 hours in IMR-32 and 22% annexin V–positive cells at 48 hours in SJ-N-KP). Selective activity of the PNA was shown by altering three point mutations, and by the observation that an antigene PNA targeted against the noncoding DNA strand did not exert any effect. These findings could encourage research into development of an antigene PNA–based tumor-specific agent for neuroblastoma (and other neoplasms) with MYCN expression.

Introduction
About 25% to 30% of primary untreated neuroblastomas present amplification/overexpression of the MYCN proto-oncogene, which is associated with advanced stage disease, rapid progression, and poor prognosis (1).

Targeted expression of MYCN in transgenic mice causes development of neuroblastoma (2). Unlike MYC, which is fairly ubiquitous and is expressed in proliferating cells, MYCN has a very restricted expression pattern (in mice, MYCN is mainly expressed during the early stages of differentiation, and in early B-cell development in adults; ref. 3). Identification of selective inhibitors of N-Myc could be relevant for development of more effective and less toxic specific therapeutic agents for neuroblastomas with MYCN overexpression. Antisense oligodeoxynucleotide–based inhibition of MYCN expression in vitro decreases neuroblastoma proliferation and promotes neuron differentiation (4). A major clinical limitation of conventional antisense oligonucleotides is that they are rapidly degraded by nucleases.

Nucleic acid–based drugs designed to overcome this limitation include peptide nucleic acids (PNA): DNA analogues in which the sugar-phosphate backbone is replaced by a pseudopeptide chain constituted by N-(2-aminoethyl)glycine monomers covalently bonded to DNA bases (5). PNAs form highly stable duplexes with complementary DNA and RNA strands, and are resistant to degradation by nucleases and proteases (6). PNAs exert antisense inhibitory effects in vitro on important tumor proteins like Pml-Rar-a and Bcl-2 (7, 8). Recently, Sun et al. (9) and our group (10) reported PNA-based antisense strategies for N-Myc inhibition in neuroblastoma cells. Interestingly, PNAs designed to target the DNA coding strand (but not mRNA) also show antigeue capacity in vitro and in vivo (even without conjugation to nuclear carriers; refs. 11–15).
Here we describe a novel sense antigen PNA conjugated with a nuclear localization signal (NLS) peptide, designed for targeted inhibition of MYCN transcription in human neuroblastoma cells, and report its effects in six cell lines: MYCN-amplified/overexpressed GI-LI-N and IMR-32 (16, 17); MYCN-unamplified/low-expressed SJ-N-KP and NB-100 (17); and MYCN-unamplified/unexpressed GI-CA-N and GI-ME-N (16). The newly developed MYCN antigen PNA-NLS can be delivered to the nucleus of neuroblastoma cells. Its inhibitory effect on MYCN transcription was highly selective and specific, leading to antiproliferative effects in neuroblastoma cells, which correlated with the rate of N-Myc expression. Compared with an antisense PNA (PNAAS) strategy for N-Myc inhibition in MYCN-amplified/overexpressed neuroblastoma cells, this antigen strategy showed stronger and longer inhibitory effect at lower concentrations. Furthermore, anti-MYC antigen induced growth arrest (with G1 phase accumulation) of MYCN-transduced cell lines at lower concentrations. Furthermore, an antisense strategy showed stronger and longer inhibitory effects in neuroblastoma cells, whereas no inhibitory effect was caused in MYCN-unamplified/unexpressed neuroblastoma control cells.

Materials and Methods

Peptide Nucleic Acid Design

The PNA was designed as homologous to a unique sequence of the noncoding (antisense) strand in exon 2 of MYCN (bp 1,650–1,665: 5'-ATGCCGGGATCATGCC-3', Genbank accession no. M13241). This PNA antigen (PNAa), which is complementary to a unique sequence in the coding DNA strand, was designed to directly inhibit mRNA synthesis. To test the specificity of the activity of this PNAa, we also designed a mismatched PNA (PNAmm) containing a 3-base substitution (homologous to 5'-GTGCCGACATGCTT-3'), the PNAas complementary to the non-coding DNA strand (and therefore also to the PNAa), and an additional antigen PNA (PNAasMYC) directed to target the MYC oncogene (belonging to the same family of MYCN). The control PNAasMYC (5'-TCAAGGTTACCTTAC-3') is complementary to a unique sequence in the exon 2 of the antisense strand of MYC, and is the same sequence used by Cutrona et al. (11) that caused a strong and specific inhibition of the MYC gene transcription in Burkitt’s Lymphoma cells.

Specificity was verified using the BLAST homology program. All the PNAs were covalently linked to their COOH terminus with an NLS peptide (PKKKRKV) to mediate transfer across the nuclear membrane (11). Fluorescently labeled PNAas-NLS was also synthesized by linking a rhodamine (Rho) fluorophore to its NH2 terminus.

Synthesis, Purification, and Characterization of Peptide Nucleic Acids

Synthesis, purification, and characterization of the PNAs were done as described (10). The PNAs synthesized are listed below:

- **PNAas-NLS**: H-ATGCCGGGATCATGCT-PKKKRKV-NH2. Crude yield: 70%. Mass spectrometry (electrospray ionization, positive ions): MH5+: calculated m/z 1,047.9, found m/z 1,047.7; MH6+: calculated m/z 873.4, found m/z 873.5; MH7+: calculated m/z 748.8, found m/z 748.9; MH8+: calculated m/z 655.3, found m/z 653.3.
- **PNAas-PNM**: H-ATGCCGGGATCATGCT-PK(KKKR)NKVN. Crude yield: 31%. Mass spectrometry (electrospray ionization, positive ions): MH4+: calculated m/z 1,313.6, found m/z 1,313.7; MH5+: calculated m/z 1,051.1, found m/z 1,051.1; MH6+: calculated m/z 876.1, found m/z 876.4; MH7+: calculated m/z 751.0, found m/z 751.2; MH8+: calculated m/z 657.3, found m/z 657.5; MH9+: calculated m/z 584.4, found m/z 584.6.
- **PNAas-NLS**: H-ATGCCGGGATCATGCT-PK(KKKR)NKVN-NH2. Crude yield: 90%. Mass spectrometry (electrospray ionization, positive ions): MH4+: calculated m/z 1,301.1, found m/z 1,301.0; MH5+: calculated m/z 1,041.1, found m/z 1,041.1; MH6+: calculated m/z 867.7, found m/z 867.8; MH7+: calculated m/z 743.9, found m/z 743.9; MH8+: calculated m/z 651.0, found m/z 651.1.
- **PNAas-PNM**: H-ATGCCGGGATCATGCT-PK(KKKR)NKVN. Crude yield: 31%. Mass spectrometry (electrospray ionization, positive ions): MH4+: calculated m/z 1,313.6, found m/z 1,313.7; MH5+: calculated m/z 1,051.1, found m/z 1,051.1; MH6+: calculated m/z 876.1, found m/z 876.4; MH7+: calculated m/z 751.0, found m/z 751.2; MH8+: calculated m/z 657.3, found m/z 657.5; MH9+: calculated m/z 584.4, found m/z 584.6.

Cells, Peptide Nucleic Acid Treatment, and Cell Growth

We used the following neuroblastoma cell lines: GI-LI-N and IMR-32, characterized by amplifications (50-fold and 20-fold, respectively) and overexpression of MYCN (16, 17); MYCN-unamplified/low-expressed SJ-N-KP and NB-100 (17); and MYCN-unamplified/unexpressed GI-CA-N and GI-ME-N (10, 16). Cell cultures were done as described (10). The PNAa-NLS was added at concentrations of 1, 5, 10, and 20 μmol/L. To evaluate the specificity of the effect of PNAa-NLS on MYCN, GI-LI-N, IMR-32, SJ-N-KP, and NB-100 cells were treated at 10 μmol/L (the selected optimal concentration for PNAas-NLS) with PNAas-NLS and PNAas-Myc-NLS (GI-LI-N, IMR-32, and with PNAas-PNM-NLS (IMR-32); MYCN-unamplified/unexpressed GI-CA-N and GI-ME-N cells were treated with 10 μmol/L PNAa-NLS. Cells were harvested and counted at 24, 48, and 72 hours after...
treatment; in the case of GI-LI-N, IMR-32, SJ-N-KP, and NB-100, the count was extended for a further 2 days. Cell count and viability were determined by the trypan blue dye exclusion method (three identical experiments).

**Cellular Uptake of MYCN Antigen PNA<sub>C</sub>-NLS**

Fluorescence microscopy analysis was performed to evaluate the intracellular localization of PNA<sub>C</sub>-NLS, GI-LI-N, IMR-32, SJ-N-KP, NB-100, GI-CA-N, and GI-ME-N cells was done by using a Rho-PNA<sub>C</sub>-NLS by the method previously described (10).

**Real-time Reverse Transcription-PCR of MYCN**

Total RNA was extracted from IMR-32, GI-LI-N, SJ-N-KP, and NB-100 using the RNeasy Mini Kit (Qiagen, Santa Clarita, CA) from cells treated and untreated with 10 μmol/L PNA<sub>C</sub>-NLS or PNA<sub>mt</sub>-NLS after 1, 6, 12, and 24 hours. Each RNA sample was quantified twice with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). First-strand cDNA was synthesized using 1 μg of total RNA and the cDNA Synthesis Kit for reverse transcription-PCR (Roche Diagnostics, Basel, Switzerland) according to standard procedures of the manufacturer.

Using an ABI-Prism 5700 (Applied Biosystems, Foster City, CA), real-time PCR was performed in triplicate using 10 ng of cDNA in a final volume of 20 μL using the SYBR Green Master Mix 2 × (Applied Biosystems; three identical experiments). Primer concentrations were 50 nmol/L for MYCN sense, 5 μmol/L; for ATPS rRNA反transcript, 300 nmol/L for ATPS primer sequences were MYCN sense, 5′-CGACCACAAGGGCCTCAGT-3′; MYCN antisense, 5′-TGACCACGCTGATTCTCTTCT-3′; ATPS sense, 5′-CTCTTCAAGGCTATGGGGA-3′; and ATPS antisense, 5′-ATGCGCTCCACATTAGAAGG-3′. PCR reaction conditions were 2 minutes at 50°C, 10 minutes at 95°C, 15 seconds at 95°C, and 60 seconds at 60°C for 50 cycles.

**Western Blot Analysis**

Western blot analysis was performed using IMR-32, GI-LI-N, SJ-N-KP, and NB-100 cells as described (ref. 10; three identical experiments). Immunodetection was done with N-Myc and β-tubulin rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) using enhanced chemiluminescence kit (Amersham Biosciences, Upplands Väsby, Sweden). Chemiluminescent bands were detected and using ChemiDoc system and quantified by the Quantity One software (Bio-Rad, Hercules, CA).

**Morphologic Analysis**

GI-LI-N, IMR-32, NB-100, and SJ-N-KP cells (2 × 10<sup>5</sup>/mL) were cultured for 48 hours in wells (from a six-well cluster plate) containing a 24 × 24 mm glass slide, in the presence or absence of PNA<sub>C</sub>-NLS (10 μmol/L; three identical experiments). Light microscopy was done using a Wilovert microscope (Hund GmbH, Wetzlar, Germany).

**Cell Cycle and Apoptosis Analysis**

Flow cytometry analysis of cell cycle and apoptosis was done as previously described (10) in IMR-32 cells (1 × 10<sup>6</sup>) at 24 and 48 hours after PNA<sub>C</sub>-NLS treatment (10 μmol/L; three identical experiments).

For apoptosis analysis in SJ-N-KP, cells were cultured in chamber slides for 48 hours in the presence of PNA<sub>C</sub>-NLS or PNA<sub>mt</sub>-NLS (10 μmol/L; three identical experiments). Staining of cells with calcein (Molecular Probes, Eugene, OR) and annexin V-biotin (Oncogene, Cambridge, CA) was done by following the annexin V-Biotin apoptosis detection kit procedure (Calbiochem, La Jolla, CA). Fluorescence microscopy analysis was done with a BX-51 microscope (Olympus, Tokyo, Japan).

**Results**

**Uptake of MYCN Antigen PNA<sub>C</sub>-NLS in Human Neuroblastoma Cells**

GI-LI-N, IMR-32, SJ-N-KP, NB-100, GI-CA-N, and GI-ME-N cells treated with Rho-PNA<sub>C</sub>-NLS (10 μmol/L) were analyzed at 30 minutes, 2.5 hours, and 24 hours (no Rho-PNA<sub>C</sub> was used as control due to its inability to enter into the nucleus without NLS; ref. 10). Fluorescence microscopy images showed that in all six cell lines, intracellular fluorescence was already detectable 30 minutes after PNA treatment. Maximum intensity was reached at 2.5 hours (Fig. 1), with the levels remaining constant until 24 hours. High intranucleic levels of the PNA were observed, alongside low levels in the cytoplasm (Fig. 1).

**MYCN Antigen PNA<sub>C</sub>-NLS Inhibits MYCN Transcription, N-Myc Production, and Cell Growth in MYCN-Amplified/Overexpressed and MYCN-Unamplified/Low-Expressed Cells**

Real-time reverse transcription-PCR of MYCN mRNA was done in MYCN-amplified IMR-32 cells (1, 6, 12, and 24 hours) and SJ-N-KP (24 hours) after treatment (10 μmol/L) with PNA<sub>C</sub>-NLS or with PNA<sub>mt</sub>-NLS. In IMR-32 at 12 and 24 hours after PNA<sub>C</sub>-NLS treatment, MYCN mRNA decreased (with respect to untreated cells) by one and two threshold cycles (50% and 75% reductions), respectively; no changes were observed after PNA<sub>mt</sub>-NLS treatment (Fig. 2A). MYCN mRNA levels were normalized to ATPS housekeeping gene levels. At 24 hours after PNA<sub>C</sub>-NLS treatment, the level of ATPS mRNA decreased (Fig. 2A); similar trends (not shown) were observed for other housekeeping gene transcripts (β-actin, β2-microglobulin, 18S rRNA, ribosomal protein L4, and GAPDH). By contrast, mRNA levels of ATPS (Fig. 2A) and other housekeeping genes (not shown) were unchanged after PNA<sub>mt</sub>-NLS treatment. Moreover, all cDNA samples had identical amounts of total RNA, as accurately quantified by the NanoDrop spectrophotometer. Thus, the housekeeping gene mRNA reductions observed, concomitant with decreased MYCN mRNA, after 24 hours of treatment with PNA<sub>C</sub>-NLS (but not after PNA<sub>mt</sub>-NLS), depended on MYCN inhibition. In SJ-N-KP, 24 hours after PNA<sub>C</sub>-NLS treatment, MYCN mRNA was consistently decreased; no changes were observed after PNA<sub>mt</sub>-NLS treatment (Fig. 2B).

N-Myc production was assessed (by Western blotting) in IMR-32 cells (at 3, 6, 12, 24, and 48 hours) and in SJ-N-KP (24 hours) after treatment (10 μmol/L) with PNA<sub>C</sub>-NLS or PNA<sub>mt</sub>-NLS. A 50% reduction of protein amount at 3 hours after PNA<sub>C</sub>-NLS treatment was followed by a progressive decrease at 6, 12, 24, and 48 hours, by which time it was not...
undetectable (Fig. 3B). By contrast, no change was observed after PNA_{mt}-NLS treatment. It should be noted, however, that at 24 and 48 hours, it was not formally possible to compare the N-Myc reduction with expression of β-tubulin and several other control proteins, due to the profound modifications in the proteome—as also revealed by Coomassie total protein staining (Fig. 3B)—caused by the early inhibition of N-Myc production. In SJ-N-KP, only treatment with PNA_{c}-NLS caused a consistent N-Myc reduction (Fig. 3C).

To evaluate the effect of PNA_{c}-NLS on cell growth, GI-LI-N and IMR-32 (MYCN-amplified/overexpressed), SJ-N-KP and NB-100 (MYCN-unamplified/low-expressed), and GI-CA-N and GI-ME-N (MYCN-unamplified/unexpressed) neuroblastoma cells were treated at concentrations of 1, 2.5, 5, and 10 μmol/L (a single-dose treatment at time 0), and cell count and viability were determined at 24, 48, 72, 96, and 120 hours. The rate of cell growth inhibition response varied with the levels of MYCN expression in the different cell lines. The MYCN-amplified/overexpressed GI-LI-N and IMR-32 cells showed consistent cell growth inhibition at the optimal concentration of 10 μmol/L. Relevant antiproliferative effects were evident at 24 hours (60% in GI-LI-N and 70% in IMR-32), increased at 48 hours, peaked at 72 hours (80% in GI-LI-N and 90% in IMR-32), and remained stable over the remaining...
2 days of monitoring (Fig. 4B). The MYCN-unamplified/low-expressed SK-N-KP and NB-100 showed less cell growth inhibition, peaking at 60% and 50%, respectively, after 72 hours (Fig. 4C). By contrast, the MYCN-unamplified neuroblastoma GI-CA-N and GI-ME-N cells showed no inhibitory effect in experiments done under the same conditions.

MYCN Antigene PNAs-NLS Is Selective and Specific for MYCN

Specificity of PNAs-NLS antigenic activity was shown by the finding that the inhibitory effect recorded in GI-LI-N and IMR-32 (MYCN-amplified/overexpressed) and SJ-N-KP and NB-100 (MYCN-unamplified/low-expressed) neuroblastoma cells was absent in GI-CA-N and GI-ME-N (MYCN-unamplified/unexpressed) cells (Fig. 4).

To test the selectivity of PNAs-NLS for its designated target in the antisense strand of exon 2 of MYCN, we also did proliferation experiments using PNAs-NLS (sequence altered by three point mutations) and in the case of GI-LI-N and IMR-32 also using PNAs-NLS (complementary to the sense strand of MYCN). No inhibitory effect was observable (Fig. 4).

Figure 3. N-Myc expression (Western blot) in IMR-32 and SJ-N-KP cells after treatment with PNAs-NLS or PNAm-NLS. Analysis of proteins extracted from untreated cells (C) or treated with PNAs-NLS (P) or with PNAm-NLS (M) at 3, 6, and 12 h after treatment (A) and at 24 and 48 h after treatment (B) for IMR-32, or at 24 h after treatment for SJ-N-KP (C). B, arrows, some of the most evident protein bands that decrease or increase after treatment with PNAs-NLS for 24 and 48 h. D, comparison of N-Myc expression in the MYCN-amplified/overexpressed and MYCN-amplified/low-expressed cell lines.

Figure 4. A, morphologic analysis of IMR-32 and GI-LI-N cells at 48 h after treatment with MYCN-PNAs-NLS. B, percentage growth rates with respect to controls after treatment of IMR-32 and GI-LI-N, with PNAs-NLS, PNAm-NLS, and PNAm-NLS. C, percentage growth rates with respect to controls after treatment of SJ-N-KP and NB-100 with PNAs-NLS and PNAm-NLS. Columns, mean of three different experiments; bars, SD.
Furthermore, we did additional control experiments by choosing an additional target gene (the MYC gene) and a specific anti-MYC PNA-NLS (PNA\textsubscript{MYC}−NLS), and we evaluated the specific antigen activity and cell growth inhibition effects in the IMR-32 cells, that while over-expressing MYCN, they also expressed MYC (Fig. 5).

PNA\textsubscript{MYC}−NLS has same sequence used by Cutriona et al. (11). PNA\textsubscript{MYC}−NLS and PNA\textsubscript{C}−NLS are complementary to a unique sequence in the exon 2 of the antisense strand of their respective target genes (MYC and MYCN).

Treatment of the IMR-32 neuroblastoma cells with PNA\textsubscript{MYC}−NLS (10 μmol/L) for 24 hours caused an inhibition in the MYC transcription (Fig. 5A) and protein production (Fig. 5B), leading to a cell growth inhibition of 60% (Fig. 5C), whereas the MYCN transcript level remained unaltered (Fig. 5A). By contrast, treatment of IMR-32 cells with the anti-MYCN PNA\textsubscript{C}−NLS led to MYCN inhibition (Fig. 5A) whereas the MYC transcript levels remained unaltered.

Morphologic analysis (Fig. 4A) of IMR-32 and GI-LI-N cells 48 hours after treatment with MYCN-PNA\textsubscript{C}−NLS (10 μmol/L) revealed that treated cells were less uniformly distributed with respect to control cells, and had the tendency to form clumps. No evident morphologic changes were observable in the MYCN-unamplified/low-expressed SJ-N-KP and NB-100 (data not shown).

**MYCN Antigene PNA\textsubscript{C}−NLS Induces Accumulation of Cells in G\textsubscript{1}**

Flow cytometric analysis in IMR-32 cells at 24 hours showed that MYCN PNA\textsubscript{C}−NLS (10 μmol/L) induced accumulations, with respect to untreated cells, of cells in G\textsubscript{1} (39–53%) and decreases in G\textsubscript{2}–M (17–6%) and S phases (45–41%; Fig. 6B). The failure to observe a sub-G\textsubscript{1} phase (relative to hypodiploid DNA content, where DNA is cleaved at the internucleosomal linker regions before death by apoptosis) indicated that cells were not in late apoptosis after 24 hours of treatment.

**MYCN Antigene PNA\textsubscript{C}−NLS Induces Apoptosis in MYCN-Amplified Cells**

Early apoptotic changes in MYCN-amplified/over-expressed IMR-32 cells were assessed by annexin V staining at 24 and 48 hours after treatment with PNA\textsubscript{C}−NLS or PNA\textsubscript{mt}−NLS (10 μmol/L). The percentage of early apoptotic cells (annexin V\textsuperscript{−}/propidium iodide\textsuperscript{−}) increased in treated cells from 2% to 56% at 24 hours, and from 6% to 36% at 48 hours (Fig. 6A). After 48 hours of treatment, the percentage of annexin V\textsuperscript{−}/propidium iodide\textsuperscript{−} cells increased from 5% to 53%, indicating a consistent amount of cells in late apoptosis.

Apoptotic changes in MYCN-unamplified/low-expressed SJ-N-KP cells were assessed by annexin V staining at 48 hours after treatment with PNA\textsubscript{C}−NLS or PNA\textsubscript{mt}−NLS (10 μmol/L). The percentage of early apoptotic cells (calcine\textsuperscript{+}/annexin V\textsuperscript{+}) was 7% in untreated cells and 10% in cells treated with PNA\textsubscript{mt}−NLS, and increased to 22% in cells treated with PNA\textsubscript{C}−NLS (Fig. 6C). Statistical analysis (test for proportion) indicated that the increase of apoptosis in SJ-N-KP was significative after treatment with PNA\textsubscript{C}−NLS ($P = 2.7 \times 10^{-3}$) whereas it was not after treatment with PNA\textsubscript{mt}−NLS ($P = 0.104$).

**Discussion**

Our *in vitro* data on human neuroblastoma cells indicate specific, selective inhibition of MYCN expression by antigen activity, leading to cell growth inhibition that varied with the rates of expression of the cell lines analyzed. Strong (80% and 90%) and persistent cell growth inhibition, G\textsubscript{1} cell cycle arrest, and apoptosis were observed in MYCN-amplified/overexpressed neuroblastoma cells; 60% and 50% cell growth inhibitions were observed in MYCN-unamplified neuroblastoma cells expressing low levels of N-Myc; no effect was observed in MYCN-unamplified/unexpressed neuroblastoma cells.

These findings suggest the possibility of developing an antigene PNA−based strategy for targeted inhibition of MYCN transcription.

PNAs can exert antigene capability *in vitro* and *in vivo* (11–15). Following interesting results reported by Sun et al. (9) and our group (10) using an antisense strategy, we developed an antigene PNA designed for targeted...
inhibition of MYCN transcription in neuroblastoma cells. For the PNA s-NLS design, we targeted a unique sequence of the antisense strand of exon 2 of MYCN (bases 1,650–1,665)—a position analogous to that chosen for an antigene PNA for MYC which led to potent specific inhibition of gene transcription and cell growth in Burkitt’s lymphoma (11). To mediate transfer to the nucleus, the NH₂ terminus of our PNA was linked to an NLS peptide (PKKKRKV). Our PNA s-NLS complementary to the antisense strand of MYCN was intended to interfere with the activity of the RNA polymerase II and MYCN transcription protein complex. In vitro (11, 12, 14, 15) and in vivo (13) studies showed that antigenic PNA functioned as sense sequences when targeted to the antisense gene strand, whereas the relative PNAas exerted a consistently less inhibitory activity (14).

In line with in vivo evidence that neuronal cells allow easy access to PNA (13), we recorded effective nuclear delivery of the Rho-PNA s-NLS in the neuroblastoma cells studied. Treatment with PNA₃-NLS (10 μmol/L) caused progressive, marked reductions of MYCN mRNA and N-Myc, leading to strong, persistent, and specific cell growth inhibitory effects in MYCN-amplified cells. The maximum effect persisting well beyond 72 hours (instead of declining after 48 hours) in MYCN-amplified and overexpressing neuroblastoma cells. The augmented inhibition likely reflects inherent advantages of antigene over antisense strategies. First, achievement of inhibitory effects at lower PNA concentrations can be ascribed to the much lower copy number of the targeted gene (even when amplified) with respect to the thousands of MYCN transcripts targeted in an antisense strategy. Second, by attacking the neoplastic clone at the root (the gene itself), an antigene strategy should allow persistence of the inhibitory effect after a single treatment (whereas an antisense strategy entails progressive subtraction of the PNA, leaving MYCN mRNA production free to continue). Our data suggest the possibility of obtaining a stronger and longer-lasting specific inhibitory effect on gene expression and avoiding the need for continuous infusion.

The increased percentage of IMR-32 (MYCN-amplified) neuroblastoma cells in G₁ phase after PNA₃-NLS treatment is in line with the knowledge on the specific role exerted by N-Myc in the cell cycle: in postmitotic sympathetic (but not cortical) neurons, high MYCN expression selectively induces S-phase reentry while protecting against apoptosis (18). Apoptosis was induced in IMR-32 as early as 24 hours after treatment.

Our PNA₃-NLS seems to be highly specific for MYCN expression and also shows a high degree of selectivity for
its designated target in the initial portion of exon 2 of MYCN sequence. Cutrona et al. (11) have suggested that specific inhibition of transcription by antigen PNAAs can probably be ascribed to invasion of the complementary double-stranded DNA, although this interpretation is still debated (19). Our data are of relevance to this discussion. The observation that a PNA bearing the same NLS element but directed against the noncoding strand is much less efficient suggests that an antisense effect can be ruled out in the interpretation of our results. The sequence specificity of the inhibition suggests that the PNA interacted with the coding strand of the DNA. Although displacement of one strand of a long double-stranded DNA with mixed sequence (i.e., non-homopyrimidine) PNAs has not been observed in vitro (20), our findings agree with other reports (11–15) suggesting that, in the nuclear compartment at least, transcriptionally active genes such as the overexpressed MYCN could be more accessible to interaction with complementary duplex-forming PNA.

PNAs belong to a third generation of nucleic acid–based gene-specific drugs. Compared with conventional oligonucleotides, they offer greater clinical potential because of resistance to degradation by nucleases and stronger binding abilities with nucleic acids. The present antigen PNA–NLS has superior potential for therapeutic molecular targeting in neuroblastoma cells expressing MYCN with respect to the reported capabilities of conventional antisense oligonucleotides (4) or PNA(15). Moreover, association of the SV40 NLS peptide with a cellular membrane transport peptide allows delivery of PNA to the nuclear compartment of living cells (11), suggesting that the present approach could readily be improved and extended to other less permeable cell lines.

Evidence supporting the feasibility of antigen PNA–based therapeutic strategies comes from in vivo animal experiments (21). Systemically injected PNA–4K oligomers (PNA with four lysines linked at the COOH terminus) exhibited sequence-specific antisense activity in various mouse organs due to favorable tissue distribution and pharmacokinetics, whereas single lysine oligomers (PNA–1K) were completely inactive, indicating that the four-lysine tail is essential for the antisense activity of PNA oligomers in vivo (22). The cationic NLS peptide conjugated to the antigen PNAAs used in our study contains four lysines and could confer similar in vivo properties.

If in vivo animal experiments confirm our in vitro results, pharmacologic interest could be aroused to develop a candidate antigen PNA–based drug designed for specific treatment of the unfavorable subset of neuroblastomas that overexpress N-Myc. A similar approach might also be considered for other tumors associated with N-Myc overexpression (3).

Acknowledgments

We thank Robin M.T. Cooke M.A. (Oxon) for writing assistance, and Dr. L. Zanella (Rizzoli Institute, Bologna) for technical assistance in cell cycle analysis.

References

Anti-gene peptide nucleic acid specifically inhibits MYCN expression in human neuroblastoma cells leading to cell growth inhibition and apoptosis

Roberto Tonelli, Stefania Purgato, Consuelo Camerin, et al.