Antitumor Activity of Sustained N-Myc Reduction in Rhabdomyosarcomas and Transcriptional Block by Antigene Therapy

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**Statement of Translational Relevance**

Rhabdomyosarcomas are the most common pediatric soft tissue sarcoma. They are a leading cause of cancer death in children with few therapeutic options in high-risk categories. N-Myc is a transcription factor belonging to the MYC protein family that has a highly restricted expression pattern in non-fetal tissues. N-Myc protein expression in patient samples of rhabdomyosarcoma and an apoptotic response to sustained reduction of expression in rhabdomyosarcoma cell lines implies that N-Myc represents a tumor-specific therapeutic target in these tumors. Systemic treatment of mice with rhabdomyosarcoma cell line xenografts using an antigene peptide nucleic acid (PNA) oligonucleotide conjugated to a nuclear localization signal peptide that inhibits transcription of MYCN shows promise by eliminating tumors. This direct antigene approach may have therapeutic potential in the treatment of other cancers and diseases.
Abstract

Purpose: Rhabdomyosarcomas are a major cause of death from cancer in childhood described with MYCN amplification and, in the alveolar subtype, transcription driven by the PAX3-FOXO1 fusion protein. Our aim was to determine the prevalence of N-Myc protein expression and the potential therapeutic effects of reducing expression in rhabdomyosarcomas, including use of an antigene strategy that inhibits transcription.

Experimental Design: Protein expression was assessed by immunohistochemistry. MYCN expression was reduced in representative cell lines by RNA interference and an antigene peptide nucleic acid (PNA) oligonucleotide conjugated to a nuclear localization signal peptide. Associated gene expression changes, cell viability and apoptosis were analyzed in vitro. As a paradigm for antigene therapy, the effects of systemic treatment of mice with rhabdomyosarcoma cell line xenografts were determined.

Results: High N-Myc levels were significantly associated with genomic amplification, presence of the PAX3/7-FOXO1 fusion genes and proliferative capacity. Sustained reduction of N-Myc levels in all rhabdomyosarcoma cell lines that express the protein decreased cell proliferation and increased apoptosis. Positive feedback was shown to regulate PAX3-FOXO1 and N-Myc levels in the alveolar subtype that critically decrease PAX3-FOXO1 levels on reducing N-Myc. Pharmacological systemic administration of the antigene PNA can eliminate alveolar rhabdomyosarcoma xenografts in mice, without relapse or toxicity.

Conclusion: N-Myc, with its restricted expression in non-fetal tissues, is a therapeutic target to treat rhabdomyosarcomas and blocking gene transcription using antigene oligonucleotide strategies has therapeutic potential in the treatment of cancer and other diseases that has not been previously realized in vivo.
INTRODUCTION

The Myc-family of transcription factors are involved in many critical cellular processes including cell proliferation and apoptosis and they are implicated in the development and progression of tumors (1). MYCN gene amplification is most widely known for its association with poor outcome in neuroblastomas (2) although other pediatric tumors including rhabdomyosarcomas (RMS) have been described with MYCN amplification (3-5). RMS are the most frequent soft tissue sarcomas in children with two main histological subtypes, Embryonal (ERMS) and Alveolar (ARMS). The majority of ARMS are associated with PAX3- or PAX7-FOXO1 fusion genes and generally more aggressive clinical behavior than ERMS (6-9). PAX3-FOXO1 fusion protein has been shown to increase transcription of MYCN (10, 11) although the prevalence of N-Myc protein expression and dependence on this in RMS has not been described.

Expression of MYCN is very restricted after birth (12) and the effects of reducing N-Myc levels in neuroblastoma cells (13, 14) has led to suggesting N-Myc as a therapeutic target for neuroblastomas. Various therapeutic strategies have been proposed, including indirect approaches that impact on the stability of the N-Myc protein (15, 16). However, therapeutic targeting of nuclear transcription factors such as those of the Myc-family is considered challenging. Oligonucleotides in clinical trials are based on antisense approaches which target mRNA and inhibit translation whereas less well known antigene strategies target the chromosomal DNA and inhibit transcription (13, 17-22). Antigene approaches offer conceptual advantages over antisense: usually two gene copies per cell to target rather than multiple copies of mRNA that are continually being transcribed; a persistence block of the transcriptional process; simultaneous inhibition of all splice variants and their simple and non-restrictive design (13, 17-22). In keeping with requirements for clinical application, peptide nucleic acid (PNA) oligonucleotides (23) show potent antigene activity (13, 17, 18, 21) and are resistant to nucleases and proteases although their in vivo potential has not been demonstrated.

Here we report the prevalence of N-Myc protein in a large series of RMS, dependence of RMS cell viability on N-Myc expression and, as a paradigm for in vivo antigene therapy, the
pharmacological activity of a MYCN specific antigene PNA to treat aggressive alveolar rhabdomyosarcomas in mice.

MATERIALS AND METHODS

RMS tissue from patients and cell lines
Formalin fixed paraffin embedded diagnostic tumor material from 79 patients with RMS was collected from UK centers through the Children’s Cancer and Leukemia Group (Local Research Ethics Committee protocol Nos. 1836 and 2015 and Multi-Regional Research Ethics Committee/06/4/71 with consent where required). The histology was confirmed by review according to WHO guidelines to be 25 alveolar and 54 embryonal and 0.6 mm diameter cores from three or more defined regions of tumor blocks were used to construct a tissue microarray. Patient data for this cohort is summarized in Table S1. A previously described tissue microarray was also available containing material from 60 alveolar and 171 embryonal cases (23). The cell lines used were, RH30, RH3, RH4, RH41, RMS, RH28, RC2, derived from ARMS which all express the PAX3-FOXO1 fusion gene except for RC2 which expresses PAX7-FOXO1. Cell lines derived from ERMS were SMS, CCA, RH36, RMS-YM, CT10 and RD. Further details of their source, validation and culture conditions are provided in the supplemental methods.

MYCN gene copy number and N-Myc immunohistochemistry analyses
Fluorescence in situ hybridization (FISH) was performed on the tissue microarrays (TMAs) and nuclei from cell lines for fusion gene status and MYCN copy number as previously described (24) using probes for MYCN (2p24) and LAF (2q11) (MP Biomedicals). The copy number of probes for MYCN (2p24) and LAF (2q11) (MP Biomedicals) were scored in at least 20 nuclei from each core and amplification defined as greater than 4 fold excess of MYCN signals relative to LAF (5) with the maximum score from all cores from a patient or cell line used. Gain of MYCN was inferred when a 1-4 fold excess of MYCN or between 4-8 copies of both probes was scored. Immunohistochemistry on the TMAs used antigen retrieval with microwave treatment in target
retrieval solution pH 6.0 (Dako) for 20 minutes. Detection used the NCMII100 antibody against N-Myc (gift from Dr Nao Ikegaki) at 1:10 dilution, Ki-67 (MIB1, 1:300 dilution, Dako), pre-diluted Universal Negative Controls (Dako) and the immPRESS™ kit (Vector Laboratories) according to the manufacturers’ instructions. Cell lines known to express N-Myc protein were used as positive controls for the immunohistochemistry on the TMAs as well as primary material and cell lines that did not (normal skeletal muscle, normal liver, RMS cell line (MYCN amplified), RD cell line (no N-Myc expression detected on western blots). Slides were only scored where convincing nuclear staining was obtained in the positive control cores, and where negative controls remained negative. Positive and negative samples ensured successful staining and each core was scored as 0 for no staining, 1 for weak, 2 for moderate and 3 for strong staining in ≥ 10% of cells for categories 1-3. Each TMA was scored independently by two pathologists (K.T., J.R-F.). In rare cases of discrepancy, a consensus score was agreed after re-analysis.

**PNA design**

A selected optimal antigene PNA (PNA-MYCN) complementary to unique sequence of the antisense strand of exon 2 was used (13). To test the specificity of PNA-MYCN, we also designed a mismatched PNA (PNA-MUT) containing a three-base substitution, and an additional antigene PNA (PNA-MYC) complementary to a unique sequence in the exon 2 of the antisense strand of MYC (16). PNAs were covalently linked to a nuclear localization signal (NLS) peptide (PKKKRKV) at their C terminus. A fluorescently-labeled PNA-MYCN was also synthesized by linking a rhodamine (Rho) fluorophore to its N-terminus. Sequence information, synthesis, purification and characterization of these PNAs is described in the supplemental methods. To evaluate the cellular uptake and intracellular localization of PNA-MYCN, fluorescence microscopy analysis was performed in RH30, RH4, and SMS cells using PNA-MYCN conjugated with rhodamine (Rho-PNA-MYCN) (10 μM) as previously described (13).
**Electrophoretic Mobility Gel Shift Assay (EMSA)**

A complementary double strand DNA oligonucleotide (MYCN<sub>WT</sub>-36mer: 5'-CACGTCCACCATGCCGGGCATGATCTGCAAGAACCC-3') was used, containing the target sequence for PNA-MYCN and flanking sequences of the MYCN gene (GENBank accession n. M13241). 4µM DNA (each strand) and 2μM PNA-MYCN or PNA-MYCN(NLS-) were incubated at 90°C for 10 min in PBS buffer (10 mM phosphate, 0.1 M NaCl, 0.1 mM EDTA) and then left to anneal at room temperature for 60 min. Gel electrophoresis used polyacrylamide (15% bisacrylamide, BioRad Criterion) in TBE 1XVoltage: 100V, run time: 100 min. Sample size: 25 mL. Staining was perfomed with silver (0,2% silver nitrate in water) at room temperature for 90 min followed by development (0,3% (w/v) sodium carbonate; 0,025% (w/v) formaldehyde, 10mg/l sodium thiosulfate), fixing (10% acid acetic, 40% ethanol) at room temperature overnight and washing with water (doubly distilled). Images were acquired using Biorad GS800 Calibrated densitometer.

**PNA treatment, RNA interference and cell line growth**

Experiments in triplicate used 96-well cluster plates, 5x10<sup>3</sup> cells were plated with 100 μl of OPTI-MEM (GIBCO) containing 4% FCS, 2mM L-glutamine and 1% penicillin/streptomycin. Cells were first incubated for 12h to permit adherence to the wells. The PNA-MYCN was added to cells at final concentrations of 1, 5 and 10 μM and cells were harvested and counted every 24h using the ATPlite assay (PerkinElmer, MA, USA).. To evaluate the specificity of PNA-MYCN for MYCN, the cells were treated at 10 μM (the selected optimal concentration) PNA-MYCN, PNA-MUT or PNA-MYC. A pool of four small interfering RNAs targeting the MYCN mRNA (siMYCN) was purchased from Dharmacon Research (sequences in supplementary methods). 5x10<sup>3</sup> cells per well were plated (96-wells plate) with 100 μl of growth medium without antibiotics to have cells 50-80% confluent at the time of transfection. The day after, for each transfection sample, siMYCN (100, 250, 500 nM and 1 μM) or scrambled (details?) was administered in 15 μl of OPTI-MEM without serum and mixed. 0,5 μl Lipofectamine (Invitrogen) was then diluted in 15 μl of OPTI-MEM without
serum and treatment performed by following the manufacturer’s instructions. Cells were then incubated at 37°C in CO₂ incubator for 6 hours prior to replacing with fresh complete medium. Cell counts and viability were determined every 24 hours, using the ATPlite assay.

In independent additional experiments using cell lines RMS, RH30, RH41 and RD, cells were plated at a density of 3x10⁴ in 24 well plates. These were transfected 24 hours after plating with 2 pools comprising 3 equally represented siRNAs (Dharmacon) (supplementary methods) at a final concentration of 33nM using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Each individual siRNA was assessed for MYCN knockdown (data not shown) and combinations of siRNAs were further optimised. Non-targeting control pool #2 (Dharmacon) was used as a negative control. Cell quantitation assays following these siRNA transfections were performed using CyQUANT® NF assay (Invitrogen) in 96 well plates and analyzed on a VICTOR² D fluorometer plate reader (PerkinElmer) using the excitation/emission spectra 485/535 nm.

Lentiviral particles for shRNA knockdown of MYCN were made by transfecting (using 60ml Lipofectamine 2000) HEK293T cells in a T75cm² flask with the lentiviral packaging vectors, pMD2.G, (3 μg) pMDLg/pRRE, (5 μg) pRSV-Rev (2.5 μg, Addgene, MA, USA) and either the MYCN shRNA vector (10 μg, TRCN0000020696, Sigma) or control shRNA vector (10 μg, SHC002, Sigma) in Optimem (Invitrogen) in a total volume of 10ml. Media was replaced on day 1 and lentiviral containing media was collected on day 2, spun to remove cell debris and the supernatant filtered through a 0.45 μm low protein binding filter (Millipore). The viral titer was calculated using the p24 ELISA (Cambridge Biosciences), viral aliquots stored at -80°C. Optimal cell plating densities were determined as 0.25x10⁶ (RH30, RD), 0.5x10⁶ (RH41, CT10), 0.6x10⁶ (RMS-YM) or 1x10⁶ (RMS) per well in 6 well plates. 24-h after plating cells were transduced at a multiplicity of infection of 5-10 in the presence of polybrene 4μg/ml (Sigma) for 24-h and after a further 24-h, selected with 1μg/ml puromycin for 3 days. Cell counts following shRNA transduction were performed using a hemocytometer in the presence of Trypan Blue.

**Generation of cell line clones overexpressing N-Myc**
The MYCN transcript was HA tagged and cloned from RMS cells into the pCI-Neo vector (Promega) using the primers MYCN F; 5'-ACCATGTACCCATACGACGTCCCAGACTACGCTATGCCGAGCTGCTCCA-3' and MYCN R; 5'-GCGAGTGACTGTCCAGTTTG-3' and fully sequenced. The pCI-Neo-HA-MYCN construct was transfected into RH3, RH4 and RMZ-RC2 cell lines using Lipofectamine 2000 (Invitrogen). Cells were trypsinized and placed into selective media containing Geneticin at 400 μg/ml 48 hrs post transfection. Cells remained in selective medium for a further 9 days at which time all untransfected cells had died under selection. Cells were then lysed with Cell Lysis buffer (Cell Signaling Technologies) and subjected to western blotting.

**Real-time RT-PCR, gene expression profiling, western blot, cell cycle and apoptosis analyses**

Total RNA was extracted from cells using the RNeasy Mini Kit (QIAGEN), reverse transcribed and gene expression levels quantified using SYBR Green or Vic and Fam labeling as detailed further in the supplementary methods. RNA was hybridized to Affymetrix HG-U133A 2.0 arrays according to the manufacturer’s protocols and analyzed as described in the supplemental methods. Western-blot analysis for N-Myc, c-MYC and MET was performed in RH30 ARMS cells using total proteins or for PAX3-FOXO1, FOXO1 and p21 analyses using nuclear proteins as also detailed in the supplemental methods. Flow cytometry analysis of cell-cycle was performed as previously described (13) in RH30 cells (1 x 10⁵) at 24h after treatment with PNA-MYCN or PNA-MUT (10 μM) (3 identical experiments). RH30 and RH4 cells (4x10⁵) were plated in slide flasks with 2 ml of OPTI-MEM (GIBCO) containing 4% FCS, 2mM L-glutamine and 1% penicillin/streptomycin. PNA-MYCN or PNA-MUT was added at 10 or 20 μM to the cells and apoptosis analysis was performed after 24 and 48 h using the *In Situ* Cell Death Detection TUNEL Kit (Roche) according to the supplier’s instructions. Fluorescence microscopy analysis was performed with a BX-51 microscope (Olympus) (3 identical experiments). Eight days post lentiviral transduction of the shRNA to reduce N-Myc in 3 ARMS and 3 ERMS cell lines, apoptosis assays were performed using Caspase3/7 Glo
(Promega) and read using a MLX™ luminometer and revelation MLX software (Dynex technologies).

**In vivo anti-tumor activity studies**

6-week-old CD1 nude mice were obtained from Charles River Laboratories. All the experiments were approved by the Scientific Ethical Committee of Bologna University (prot. N° 47621-x/6 and prot. N° 46903-x/6) and conducted in the CRBA (www.CRBA.it). A total of $7 \times 10^6$ RH30 cells growing at logarithmic phase were centrifuged and resuspended in 100 µl of physiological solution. Cell suspensions were injected subcutaneously in the dorsal tissue with an insulin syringe under anesthesia with Zoletil 50 (10 µl i.m.). This xenograft murine model of ARMS was monitored by real-time molecular imaging by small animal PET (microPET) analysis, in accordance with our previous optimization study (25). Animals underwent one 18F-fluorodeoxyglucose (FDG) (microPET) scan two days after the inoculum of tumor cells and according to our previous experience (25), those found negative were excluded from the protocol because of a low probability to engraft. Semi-quantitative analysis was carried out for each tumor identified using the target to background ratio (TBR) as previously described (25).

Mice were treated after 10 days from inoculation of the tumor cells, when the tumors reached the tumor mass of approximately the 100 mm³ in size. Three treatment groups of mice were delineated: a first group (8 mice) was treated with PNA-MYCN, a second group (4 mice) with PNA-MUT and a third group (13 mice) was treated in the same way with placebo (physiological solution). Mice were injected intraperitoneally (IP) at the concentration of 50 mg/kg of PNA-MYCN or PNA-MUT in 100 µl of physiological solution alternatively in the right and in the left side of abdomen. The administration schedule consisted in injections every 48 h. The total treatment lasted 20 days. Food intake, body weight and general behavior were noted during all the study.

**Toxicological analysis**

For the toxicological analysis of the PNA-MYCN treatment *in vivo* two mice 129 svJ/X1 were obtained from our colony (Authorization from Ministry of Health Prot.1237/p) and maintained in the
same conditions described for CD1 nude mice used for the anti-tumor analysis. The toxicological analysis was made administrating PNA-MYCN on two immunocompetent mice (129 svJ/X1) versus two mice treated with physiological solution. PNA-MYCN was injected following the same schedule described above. During and before treatment mice were kept in standard conditions, weight was monitored and the site of infection was kept under observation for any skin irritation. Mice were sacrificed 24 hours after the last PNA-MYCN injection and a complete necropsy was performed with the histological evaluation of the more relevant organs.

To evaluate the bone marrow cells, bone marrow touch was performed while peripheral blood was collected. Both were stained with May-Grünwald-Giemsa staining. Free antigen PNA-MYCN in serum was determined by HPLC-MS/MS as detailed in the supplemental methods.

Statistical analyses
Correlations between copy number and immunohistochemistry scores were assessed by Chi squared for trend analyses. Correlations with Ki67 were assessed comparing no expression with any expression using Fisher’s exact test and overall survival, where data was available, was investigated using Kaplan-Meier analyses. Tumor to Background Ratio (TBR) at the first scan was compared with TBR at the second, third and fourth scans (t test). Statistical significance between experimental values was determined by t testing, and differences were considered significant if p<0.05.

RESULTS

N-Myc protein is frequently expressed in rhabdomyosarcomas and high levels are associated with amplification, fusion gene status, poor outcome and proliferative capacity

We first determined the prevalence of N-Myc protein in ERMS and ARMS. Immunohistochemistry (IHC) for N-Myc protein was positive in 55% of all RMS, 76% (41/54) of fusion gene positive ARMS and 45% (70/155) of ERMS (Fig. 1, Table 1 and Supplementary Table S2). FISH analysis
determined the MYCN copy number in the primary samples (Fig. 1). A correlation between the MYCN copy number and IHC scores was significant in ARMS and ERMS samples (Chi squared for trend p=0.039 and p<0.0001, respectively) and in ARMS the frequency of amplification was 25%, similar to that previously reported by us and others (3, 5). Fusion gene positive ARMS were associated with higher N-Myc expression compared with ERMS (Chi squared test for trend, p<0.00005) and higher expression was associated with poorer clinical outcome in patients with ARMS (Log rank test, p=0.012, n=48) by Kaplan Meier analysis but was not significant within fusion positive (Log rank test, p=0.07, n=39) or ERMS cases (Log rank test, p=0.52, n=149). N-Myc expression was positively associated with the marker of proliferation, Ki-67, in both ARMS and ERMS (Fisher’s Exact test, p=0.024 and p=0.0028, respectively)) (Supplementary Table S3). Therefore N-Myc protein is frequently expressed in RMS and high levels are associated with amplification, PAX3/7 FOXO1, poor outcome for samples with alveolar histology (irrespective of fusion gene status) and proliferative capacity.

To characterize cell lines for the functional studies, FISH analyses was also used to assess MYCN copy number (Fig. 1). The cell lines RH30, RMS, RH28 (with PAX3-FOXO1) and RC2 (with PAX7-FOXO1) had genomic amplification of MYCN, RH30 in a heterogeneous manner. The RH41 and RH4 lines (with PAX3-FOXO1) and the embryonal cell line CT10 had MYCN gain and the cell lines RD, RMS-YM, CCA, RH36, SMS and RH3 had normal copies of MYCN.

Antigene PNA-MYCN causes potent and specific inhibition of MYCN transcription and translation

We then synthesized an optimal antigene PNA (agPNA) that specifically blocked MYCN transcription (13). MYCN has three exons (22) but as exon-1 has many transcriptional start sites (26) it was not appropriate to design agPNAs targeting these (20, 21). We selected an optimal sense agPNA (PNA-MYCN) targeting a unique sequence of MYCN gene in exon-2. As PNA-MYCN is a sense sequence complementary to the MYCN antisense strand target sequence, its activity can only occur by inhibiting transcription. The anti-MYCN agPNA was conjugated to a nuclear localization signal peptide (NLS) for delivery (13, 17, 18) that we show penetrates cells
without requiring a transfection agent and localizes to the nucleus (Fig. 2A). Moreover, the EMSA assay performed on a 36mer dsDNA containing the target sequence showed that only the PNA conjugated to NLS is able to bind the target double strand DNA (Fig. 2B), consistent with the critical role for the NLS peptide in double strand DNA invasion (13, 17, 18, 27). PNA-MYCN caused a potent block of MYCN transcription in RH30 alveolar cells that reduced protein levels (Fig. 2C,D).

**MYCN silencing by antigen PNA-MYCN causes specific cell-growth inhibition and apoptosis in rhabdomyosarcoma cells**

The effects of silencing MYCN using PNA-MYCN were then assessed in cell lines representative of ARMS and ERMS. The specific block of MYCN transcription and reduction of N-Myc protein by PNA-MYCN (Fig. 2C) was followed by cell-growth inhibition. The level of cell-growth inhibition (Fig. 3B) positively correlated with basal MYCN expression levels (Fig. 3A)(Pearson's correlation coefficient, $r^2=0.88$, $P=0.0005$). G1 cell cycle arrest and apoptosis was also demonstrated in ARMS cells (Fig. 3C and 3D). The lack of effect of PNA-MUT (a control PNA containing three point mutations in the PNA-MYCN sequence) on MYCN levels (Fig. 2C) and on the phenotype of cell lines (Fig. 3B) as well as no effect of PNA-MYCN in RD (Fig. 3B), which has no MYCN expression, is consistent with specificity. PNA-MYCN without the NLS peptide (PNA(NLS-)) did not inhibit MYCN transcription (Supplementary Fig. S1A) or cell-growth (data not shown), demonstrating that the NLS is essential for the antigene activity.

**In vitro validation of MYCN as a therapeutic target using RNA interference**

In order to further demonstrate that loss of cell growth and viability was specific to N-Myc reduction, expression was reduced by RNA interference in multiple RMS cell lines with and without MYCN amplification. Cell numbers were reduced by transient reduction of MYCN using 3 independent pools of siRNAs (Supplementary Fig. S2 and S3) and knockdown using a lentiviral construct to deliver an shRNA. The sustained level of reduction by shRNA achieved (Fig. 4A,B) eliminated cells in all MYCN expressing cell lines including ERMS cell lines (Fig.4C). This resulted
in 3 to 8 fold increases of Caspase 3/7 activity in all cells lines tested except RD, which does not express MYCN (Fig. 4D). These results using shRNA knockdown were similar to the effects of PNA-MYCN although the time-course for PNA-MYCN was shorter than those for shRNA MYCN reduction (Fig. 3B). In contrast, the siRNA approach inhibited cell growth but was unable to eliminate tumor cells, even with a daily treatment regimen (Supplementary Fig. S2 and S3).

Gene expression changes associated with MYCN silencing and regulation of PAX3-FOXO1 expression by MYCN in ARMS cells

Global gene expression profiles of ARMS cells (RH30) after 12 hours of PNA-MUT treatment compared to untreated cells showed strong similarities (pearson R² = 0.920) consistent with few off-target effects. PNA-MYCN did not alter expression of the homologous MYC gene (Fig. 5A,B) and the specific silencing of MYC expression by an antigene PNA (PNA-MYC) in ARMS cells did not affect MYCN expression (Supplementary Fig. S4). This shows that the antigene PNA used were specific for each MYC gene family member. However, PNA-MYCN treatment induced differential gene expression (pearson R² 0.751 and 0.73 vs PNA-MUT and untreated cells, respectively) that gene ontology analysis related to key features of RMS biology, including cell proliferation and myogenesis (Supplementary Tables S4 and S5). Validation of differentially expressed genes was performed by quantitative RT-PCR (Fig. 5A) and western blotting (Fig. 5B). Surprisingly, reducing N-Myc levels affected genes previously associated with expression of PAX3-FOXO1 or known direct downstream targets of PAX3-FOXO1 (6, 7, 10, 28-30) such as MET, a direct target of PAX3-FOXO1 (30) and p21CDKN1A/CIP1, that is linked to PAX3-FOXO1 oncogenicity (31) and G1 arrest (Fig. 3C, 5A,B). Consistent with this, N-Myc reduction decreased expression levels of the fusion genes (Fig. 5A,B), but not PAX3 or PAX7 (Fig. 5A). Conversely, overexpression of HA-N-Myc in appropriate cell line increased levels of PAX3-FOXO1 and PAX7-FOXO1 protein 11 days post-transfection (Fig. 5C). Taken together with evidence for PAX3-FOXO1 regulating the transcription of MYCN (7, 10, 28, 29), our data are consistent with positive feedback mechanisms contributing to levels of these proteins.
**Antigene PNA-MYCN causes specific eradication of ARMS cells in mice**

Finally we evaluated the *in vivo* anti-tumor activity of PNA-MYCN in a xenograft murine model of ARMS by real-time molecular imaging using microPET, with FDG as a probe (25). Treatment of mice with PNA-MYCN led to tumor elimination in 75% of cases, and strongly reduced the tumor signal in the other 25% (Fig. 6). The TBR was significantly different for PNA-MYCN (*p*=0.018) but not PNA-MUT (*p*>0.05), both compared to the placebo. These *in vivo* results were obtained without the need for continuous treatment, but systemic administration (intraperitoneal (IP), 50 mg/kg) every two days for 20 days. Subsequent monitoring for 30 days by microPET, did not show any reappearance of the tumor in all 75% of cases where treatment had led to elimination and showed stabilization of the minimal residual tumor mass in the remaining 25% of mice. MYCN is hardly expressed in non-fetal mouse and human tissues and can be considered tumor-specific. Preliminary toxicological analysis of the human PNA-MYCN, in immunocompetent mice after the same treatment schedule did not reveal any signs of general toxicity (Supplementary Fig. S5). Pharmacokinetic analysis showed persistence of the intact PNA-MYCN, including the delivery NLS peptide, up to 12 hours after injection (Supplementary Fig. S6).
DISCUSSION

In this study we have demonstrated that N-Myc, with its restricted expression in non-fetal tissues, represents a potential tumor-specific therapeutic target in RMS. Despite this transcription factor generally being considered a challenging target, we provide evidence that antigene therapy targeting MYCN transcription has potential in treating tumors in vivo through systemic administration of MYCN specific antigene PNA. We show that N-Myc protein is expressed in over half of RMS samples in a large cohort. High levels significantly correlated with an increase in copy number of MYCN and/or the presence of the PAX3-FOXO1 or PAX7-FOXO1 fusion genes. The correlation of N-Myc expression with clinical outcome in cases with alveolar histology is consistent with fusion-gene positive cases, that express N-Myc more highly, generally having a poorer outcome than fusion gene negative alveolar cases (6, 7, 9). Seventy seven percent of fusion positive samples were immunohistochemically positive for N-Myc and all cases with the fusion gene that we have previously tested by quantitative RT-PCR show some level of MYCN expression (5)(and data not shown). Therefore a high proportion of RMS samples have the N-Myc target present.

Both N-Myc and the fusion proteins are known to play pivotal and co-operative roles in the biology of ARMS (7, 10, 11, 29, 32, 33). An association of N-Myc expression with fusion gene positivity is consistent with previous studies that have shown MYCN is a direct downstream target of the PAX3-FOXO1 fusion protein (7, 10, 28, 29). However, further to this, we have shown that N-Myc modulation clearly alters expression of PAX3-FOXO1 and PAX7-FOXO1 at the RNA and protein levels and affects known downstream targets of PAX3-FOXO1. These include MET, a direct target of PAX3-FOXO1 (but not N-Myc), that decreases in expression levels after N-Myc reduction and is known to play an important role in RMS development in mice (34, 35) and is associated with tumor invasiveness (36, 37). N-Myc regulation of PAX3 promoter activity has been shown to involve a noncanonical E box site in the 5' promoter region of PAX3 (38). However, we have shown N-Myc reduction decreased fusion gene expression without changes to PAX3 levels. This implies that regulation of PAX3 and fusion gene expression levels by N-Myc is complex and regulation of PAX3 by microRNAs has recently been described for PAX3 (39, 40).
Silencing MYCN expression induced and repressed a number of cancer-related genes linked to gene ontology features including myogenesis and cell proliferation. For example, PLK4 (Polo-like-kinase-4), a major regulator of centriole formation and associated with centrosome amplification (41, 42), decreased. This is consistent with the report that PLK4 and MYCN are overexpressed in PAX3-FOXO1-positive ARMS from transgenic mice (43). The p53 pathway is frequently inactivated in RMS through somatic mutations or functional inactivation (44). Remarkably, MYCN silencing restored p53-independent transcriptional regulation of major p53 downstream target genes in ARMS, both in terms of repression (such as PLK4 that is strongly repressed by p53 in tumors (45)) and activation (such as p21, BTG2 and GDF15). In all RMS cell-lines, reduction of N-Myc caused upregulation of p21CDKN1A/CIP1, a known repressive transcriptional target of Myc (46). N-Myc promotes progression from G(1) to S phase of the cell cycle, while p21 promotes G(1) arrest which is consistent with our observation that N-Myc reduction caused an accumulation of cells in the G(1) phase.

The apoptotic responses observed in RMS cell lines after sustained inhibition of N-Myc are similar to that reported for neuroblastoma derived cells (13, 14) as well as the apoptosis following inhibition of PAX3-FOXO1 expression in ARMS cell lines (47). In this study we adopted an antigen strategy to persistently inhibit transcription of MYCN, that is induced by PAX3-FOXO1 protein in ARMS (10, 11). An antigen approach targets the gene in contrast to antisense that inhibits continually transcribed mRNA species. We have demonstrated that the PNA-MYCN antigen fulfils many requirements of an effective drug candidate including specificity, cellular and nuclear uptake, with the nuclear localization signal peptide also enhancing the stability of the PNA:DNA duplex (19), allowing the PNA-MYCN to strand-invade the target DNA sequence. Bioavailability at 12 hours after injection and lack of toxicity was also demonstrated in mice in our investigations, although more extensive analyses will be required to comply with regulatory bodies. The minimum time to obtain a major MYCN mRNA decrease using the PNA-MYCN was shorter than shown by RNA interference approaches which may be explained by the fact that the transcriptional block by the antigen PNA-peptide does not require the activation of protein complexes involved in mRNA target degradation used in antisense approaches, such as the RISC.
complex or RNAse-H. Furthermore, silencing of MYCN by antigene PNA-MYCN led to complete tumor cell loss in vitro through PNA-MYCN administration only every three days, while treating with siMYCN required daily treatment that was unable to eliminate all tumor cells. Similarly, a non-continuous administration (every two days) in vivo with antigene PNA-MYCN eliminated tumors in a previously validated xenograft model (25). The higher efficacy and persistence of the pharmacologic effect observed by the antigene approach suggests that from a therapeutic perspective fewer doses than antisense would be required.

Complex algorithms based on mRNA structures are generally required to make effective antisense oligonucleotides. The optimal design of potent and specific antigene PNA oligonucleotides that will inhibit expression of mRNA splice variants appears relatively simple (13, 18, 21), for example the design of an antigene PNAs that effectively targets a transcriptional start site of the human progesterone receptor (21). The unique properties of the antigene approach are a potential alternative or complementary strategy to antisense and conventional pharmaceutical approaches. The dependence of RMS cells on MYCN expression demonstrated here that was effectively targeted by PNA-MYCN both in vitro and in vivo shows promise for the treatment of clinically aggressive RMS and other tumors.

ACCESSION NUMBERS

Gene expression data has been deposited in the GEO database, accession number GSE7959.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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constructive criticism of the manuscript. We acknowledge NHS funding to the NIHR Biomedical Research Centre. R.T. and J.S. designed the study and jointly wrote the manuscript. R.T. supervised experiments related to the PNAs and some of those involving siRNAs. J.S. oversaw all other investigations.

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**REFERENCES**


FIGURE LEGENDS

Figure 1. MYCN copy number in primary rhabdomyosarcoma (RMS) and representative cell lines and immunohistochemistry for N-Myc in tumor samples. MYCN copy number was assessed by FISH analysis of nuclei (blue) where the signal for the MYCN probe at 2p24 is red and the control probe (LAF) at 2q11 is green, 1 micron scale bar. Parallel MYCN expression was assessed by immunohistochemistry (brown), 10 micron scale bar.

Figure 2. Anti-MYCN antigen PNA-NLS causes potent and specific inhibition of MYCN transcription and translation. A. Representative fluorescence microscopy analysis of PNA-MYCN uptake by RMS cells (RH30, RH4 and SMS) treated for 2 hours with Rho-PNA-MYCN. B. Electrophoretic gel shift assay (EMSA) of dsDNA showing strand invasion by antigen PNA-NLS. Lanes from the left to the right: 50bp marker; dsDNA (36mer containing the MYCN target sequence for PNA-MYCN); ssDNA 36mer containing the target sequence plus PNA-MYCN,
without NLS; ssDNA 36mer containing the target sequence plus half equivalent of PNA-MYCN, showing the mobility of the DNA:PNA-NLS duplex; dsDNA plus PNA-MYCN without NLS; dsDNA plus PNA-MYCN. C. Expression of MYCN mRNA assessed by real-time RT-PCR in MYCN-amplified ARMS cells (RH30) after treatment with PNA-MYCN or PNA-MUT (mean of three separate experiments; bars, Standard Deviation (SD)). D. Representative western-blots of experiments showing N-Myc expression in MYCN-amplified ARMS cells (RH30) after treatment with PNA-MYCN or PNA-MUT.

**Figure 3.** Antigene PNA-MYCN causes specific cell-growth inhibition in RMS cells that correlates with the MYCN expression levels. A. Comparison of basal MYCN expression in ARMS and ERMS cell-lines: mRNA on the top (columns are the mean of three Real-time RT-PCR experiments; bars, SD) and protein on the bottom (representative western-blot from three identical experiments). B. Percentage cell-growth inhibition rates after treatment (every three days) with 10 μM PNA-MYCN or PNA-MUT, with respect to untreated cells. Columns show the mean of three different experiments; bars, SD. (Bottom) Prolonged administration (every three days) in both ARMS (RH30) and ERMS (CCA) cells. C and D. PNA-MYCN induced G1 cell-cycle arrest (Student's t-test, PNA-MYCN vs CTRL p<0.05) (C) and apoptosis (D) in MYCN-amplified ARMS cells (RH30)(experiments performed in triplicate). For the cell cycle analysis (C) the SD for the control, PNA-MUT and PNA-MYCN were equivalent to: G1 3.7%, 4.3%, 4.9%; S 2.7%, 2.2%, 3.4%, G2/M, 1.9%, 2%, 1.9%, respectively. In figure D, *** indicate a student t-test with P<0.0001 both vs. control and PNA-MUT.

**Figure 4.** Effect on cell number of N-Myc knockdown by lentiviral shRNA in rhabdomyosarcoma cell lines. Cell lines RMS, RH30, RH41 represent the alveolar subtype and RD, RMS-YM and CT10 the embryonal subtype with no expression of N-Myc detected in RD. Reduction of N-Myc protein as shown by immunoblotting (A), and MYCN RNA as shown by real-time PCR analysis (B) affects cell viability (C). Cell lines were plated on day 1, transduced with lentiviral shRNAs day 2, selected with puromycin on day 4 and cells counted on days 7, 9, 11 and 14. Error bars refer to
standard deviation from the mean (SD) of 4 replicate assays from a representative experiment. Light microscopy pictures of cells stained with crystal violet were taken just before harvesting the cells on the final day, scale bar 50 microns. D. Lentiviral shRNA N-Myc reduction induced apoptosis in both alveolar and embryonal cell lines that express the protein. Black and grey bars represent caspase activity associated with shRNA and control shRNA, respectively, with SD of 4 replicate assays from a representative experiment shown.

**Figure 5.** Antigene PNA-MYCN causes specific gene expression changes in RMS. A. Validation of gene expression changes by quantitative RT-PCR analysis after treatment with PNA-MYCN, siMYCN or PNA-MYC of RMS cell-lines. The treatment with the PNA-MUT showed results comparable to the untreated cells for all the genes, and thus are not presented. Each square represents the difference (Log Ratio) between untreated and treated cells. The squares filled with diagonal lines are unexpressed genes. B. Representative western-blots for PAX3-FOXO1, MET, c-Myc and p21 after the PNA treatment of RH30 cells. C. Representative western blots for N-Myc and PAX3-FOXO1 in cell line clones for RH3, RH4 and RC2 either negative or positive for overexpressing HA-MYCN.

**Figure 6.** Antigene PNA-MYCN causes specific elimination of alveolar RMS (ARMS) in mice. A. Tumor to Background Ratio (TBR) time-curve of the 18F-fluorodeoxyglucose (FDG) microPET analysis in CD1 nude mice treated with PNA-MYCN (8 mice) or PNA-MUT (4 mice) or placebo (13 mice); the treatment (IP, 50 mg/kg) was administered once every two days for twenty days. *P* value is compared to placebo. B. Representative microPET analysis at the end of the treatment schedule (20th day) in mice treated with physiologic solution (placebo) or PNA-MUT or PNA-MYCN.
Table 1. Frequency of *MYCN* copy number changes determined by FISH and N-Myc expression by immunohistochemistry of primary alveolar (ARMS) and embryonal (ERMS) patient samples in tissue microarrays.

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Antitumor Activity of Sustained N-Myc Reduction in Rhabdomyosarcomas and Transcriptional Block by Antigene Therapy

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