SPANDIDOS PUBLICATIONS

High level MycN expression in non-*MYCN* amplified neuroblastoma is induced by the combination treatment nutlin-3 and doxorubicin and enhances chemosensitivity

SUSAN K. PEIRCE and HARRY W. FINDLEY

Department of Pediatrics, Division of Hematology and Oncology, Emory University School of Medicine, Aflac Cancer Center and Blood Service, 2015 Uppergate Drive, Atlanta, GA 30322, USA

Received March 31, 2009; Accepted June 26, 2009

DOI: 10.3892/or_00000586

Abstract. *MYCN* gene amplification is a negative prognostic indicator in neuroblastoma and high level MycN expression in Stage IV neuroblastoma is generally a hallmark of poor patient outcome. However, high level expression of the MycN protein in neuroblastoma cells lacking *MYCN* amplification suppresses growth and drives apoptosis; this, in part, explains the absence of clinical observations of high level MycN in neuroblastoma lacking *MYCN* amplification. In the current study, we found that combination treatment with nutlin-3 and doxorubicin upregulated MycN expression in non-*MYCN*-amplified neuroblastoma cells at both the protein and mRNA levels. The induced expression of MycN in non-*MYCN*-amplified cells inhibited cell proliferation and increased apoptosis. MycN induction also upregulated p53, p21 and Bax protein levels, as well as mRNA levels for the positive neuroblastoma prognostic factors CD44 and EFNB3. Blocking MycN reversed these effects. These results were corroborated by findings using a MycN-inducible system in SHEP cells, another *MYCN* non-amplified neuroblastoma cell line. Our results indicate that doxorubicin/nutlin-3 combination treatment both induces expression of MycN in a non-*MYCN*-amplified background and sensitizes neuroblastoma cells to chemotherapy. These findings support the idea that induction of MycN in non-*MYCN*-amplified cells drives neuroblastoma cells toward apoptosis and suggest that combination nutlin-3/doxorubicin treatment may be clinically important.

Introduction

Neuroblastoma (NB) is a childhood solid tumor of the peripheral nervous system arising from neural crest progenitor cells and the most common pediatric solid tumor in children under five years of age; it is thought to arise from improper differentiation of neural crest cells. One of the many enigmas of NB is that most infants experience complete regression of the disease even without therapeutic intervention, while older children have a far more unfavorable outcome, with metastatic disease that is often unresponsive to intense therapy. In addition, initial responsiveness to chemotherapy may sometimes lead to relapse and drug resistance (1).

In addition to younger age, there are other favorable prognostic factors, including lack of *MYCN* amplification (2), early tumor stage and expression of the genes EPHB6, EFNB2 and EFNB3 (3); these are members of the ephrin family, receptor protein-tyrosine kinases involved in the development of the nervous system. Expression of the cell-surface glycoprotein CD44 has been found to be a statistically reliable independent predictor of event-free survival (2) and aggressive NB tumors with *MYCN* amplification have been shown to downregulate the expression of CD44 (4). High level expression of EPHB3, EFNB2, EFNB3 and CD44 correlate with low-stage NB (5) and forced expression of these genes inhibits the growth of NB cells (3). Additionally, expression of EPHB6, EFNB2 and EFNB3 genes correlates with high levels of the neurotropin receptor TrkA, another favorable prognostic factor (6). Thus, these molecular markers can be used in combination with other factors in a predictive model of favorable NB outcome.

In contrast, the deregulated high level expression of MycN in conjunction with *MYCN* amplification is prognostic of poor patient outcome in NB (reviewed in ref. 7). The expression of MycN is essential during normal neural crest development, but it is downregulated as tissues terminally differentiate (8). The critical E2F-1, 2, 3 and 4 transcription regulators bind in the autoregulatory site of the MycN promoter and have been shown to upregulate or repress MycN expression (9), in addition to factors that alter the chromatin states of the MycN promoter (10). The role *MYCN* amplification plays in NB tumorigenesis was confirmed in a pivotal early study using transgenic mice that overexpress MycN in neuroectodermal cells and develop neuroblastoma (9). In the context of NB malignancy, MycN is a transcription factor that heterodimerizes with Max to interact with E-box elements (10) within promoters of genes generally associated with proliferation and drug resistance, thus upregulating expression of proteins such

Correspondence to: Dr Susan K. Peirce, Department of Pediatrics, Division of Hematology and Oncology, Emory University School of Medicine, Aflac Cancer Center and Blood Service, 2015 Uppergate Drive, Atlanta, GA 30322, USA
E-mail: susan_peirce@oz.ped.emory.edu

Key words: neuroblastoma, nutlin-3, MycN

as MRP1 (11) and insulin-like growth factor (12). In this manner, MycN has been shown to enhance NB cell growth and tumorigenicity. *MYCN* amplification also correlates with a failure to arrest in G1 (13). A number of studies have linked coexpression of high MycN levels with high level expression of other negative prognostic NB genes (14), including pAkt (15), VEGF (16), livin (17) and MDM2, the p53 regulatory gene which has been found to be a transcriptional target of MycN (18). Conversely, the knock-down of MycN expression in some *MYCN*-amplified NB cells is associated with caspase-3 activation and apoptosis (19) as well as neuronal cell differentiation (20).

However, the prognostic significance of MycN over-expression in NB appears to depend on *MYCN*-amplification status. A number of studies have shown that high level MycN expression in the absence of amplification is associated with a favorable outcome (5,21,22,24). Indeed, conditional expression of MycN in some non-*MYCN*-amplified NB cell lines has been shown to significantly inhibit cell growth and to induce apoptosis (23,26). Conversely, in another non-amplified NB cell line, it was observed that neither forced expression of MycN nor the use of cytotoxic drugs induces apoptosis, but that the two interact synergistically (25) and that this response is dependent on mitochondrial permeability and the activation of caspases. Another study supporting the role of caspases in MycN-induced apoptosis includes the observations that *MYCN* amplification is often independently associated with methylation of caspase-8 and with defects in tumor factor-related apoptosis inducing ligand (TRAIL); ectopic MycN expression in non-*MYCN*-amplified cell lines induces both caspase-8 and caspase-9-mediated apoptosis (26).

In this study, we initially investigated the ability of nutlin-3 to increase sensitivity to doxorubicin in a non-*MYCN*-amplified NB cell line, SH-SY5Y. We demonstrate marked induction of MycN in SH-SY5Y cells at both mRNA and protein levels following combination treatments. Significantly, MycN induction was accompanied by increased sensitivity to doxorubicin in the presence of nutlin-3. This interaction was corroborated using a MycN-inducible cell system in conjunction with combination treatments. Our findings suggest a novel and distinct pathway involved in chemotherapy-induced NB apoptosis. Our results also extend previous findings showing that chemotherapy in combination with forced expression of MycN in non-amplified *MYCN* NB cells induces apoptosis (25,27).

Materials and methods

Reagents and primers. Nutlin-3 (cat. no. 10004372) was purchased from Cayman Chemical (Ann Arbor, MI) and represents an equimolar mix of the active (nutlin-3a) and inactive (nutlin-3b) isomers. In this study, all molar concentrations are expressed in terms of nutlin-3a. Doxorubicin was obtained from the pharmacy of Egleston Hospital of Emory University. EFNB3-specific forward and reverse primer sequences are: 5'-ctgaaatgcccatggaaaga-3'; and 5'-acgcccagc aagagcagcgc-3'. The forward and reverse primer sequences for CD44 are: 5'-ccgctatgtccagaaaggag-3'; 5'-tccagggactgtctt cgtct-3'. MycN forward and reverse sequences are: 5'-cggtcc cccacctctctt-3'; 5'-cggtttagccaccaactttctc-3'.

Cell lines, light microscopy and siRNA silencing. SH-SY5Y cell lines were maintained in RPMI-1640 medium + 10% FBS. Cells were photographed with an Olympus IX50. The SHEP Tet21N MYCN expression system previously described (28) was used to conditionally express MycN in a non-MycN-amplified background. Cells were grown in RPMI-1640 containing hygromycin at 25 μg/ml and supplemented with 10% tetracycline-free FCS (Clontech, Mountainview, CA). To block expression of MycN, 10 ng/ml of tetracycline was added to growth media for at least 24 h. siRNA (Stealth Prevalidated cat. no.46454497) for MycN knock-down and the Stealth Negative Control (cat. no. 12935) were purchased from Invitrogen Life Technologies (Carlsbad, CA) and the target sequence for MycN is: 5'-ccacgugccggaguugguaaa gaau-3'. Cells were plated 24 h before transfection at a concentration of $2x10^4$ per mm well (96-well plate) or $3x10^5$ per 35 mm^2 well; siRNA or the negative control were transfected using Oligofectamine (Invitrogen; cat. no. 12252) at 5 pmole (96-well plate) or 200 pmole (35 mm well), per manufacturer's instructions. Cells were assayed at 24, 48 and 72 h post transfection.

Western blotting and antibodies. For Western blotting, cells were lysed in buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM ß-glyceraldehyde, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF. Thirty-fifty μg/well of cleared lysate were run on 10 or 12.5% Tris/glycine PAGE gels and transferred to NC under standard conditions. The following primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-p53 FL-393 (sc-6243), anti-Bax (sc-20067), anti-E2F1 (SC-193) and anti-p21 (sc-817). Phospho-MDM2 (#3521) was purchased from Cell Signaling Technology (Beverly, MA). Anti-ß-actin (AC-74) was purchased from Sigma (St. Louis, MO). Anti-N-Myc antibody (OP-13) was purchased from EMD Calbiochem (Darmstadt, Germany). All primary antibodies were used at manufacturer's suggested concentrations. Goat anti-mouse or rabbit HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) were used at 1:2000.

Real-time RT-PCR. A two-step real-time RT-PCR procedure was used. All reagents were purchased from Qiagen (Valencia, CA) and all methods used were those recommended by the manufacturer. Total RNA was isolated from cells using QIAshredders (cat. no. 79654) and RNeasy (cat. no. 74104). For RT, samples were incubated at 42˚C using Qiagen Quanti Tec RT reagents. SYBR-Green reagents (cat. no. 204143) were used for the 40 cycle real-time PCR and reactions were run on an Applied Biosystems 7500 Real-Time PCR machine. GADPH was used to normalize levels of mRNA for the relative quantification method of analysis.

Cell proliferation and apoptosis. The tetrazolium-based colorimetric assay, WST-1 (cat. no. 11644807, Roche Applied Science, Mannheim, Germany), was used for the quantification of cell proliferation. Cells were plated at $2x10^4$ cells/well of a 96-well plate and treatments were performed in triplicate. Measurements were made at 450 nm with a reference wavelength of 600 nm. The Cell Death Detection ELISA^{PLUS} kit

11774425, Roche Applied Science) was used to measure levels of mono- and oligonucleosomes in the cytoplasm of apoptotic cells after treatments, following the manufacturer's instructions, with the following modification: 10^3 cells were used per well of a 96-well plate. Measurements were made at 405 nm with a reference wavelength of 490 nm.

Statistical analyses. All experiments were repeated independently at least three times. Where shown, results are presented as mean ± SE. Statistical analyses were conducted using Student's t-test. Group differences resulting in P-values of <0.05 were considered to be statistically significant. *P<0.05, **P<0.01, ***P<0.005.

Results

Nutlin-3 and doxorubicin interact to inhibit cell proliferation, induce apoptosis and substantially upregulate the expression of MycN in SH-SY5Y cells. To gain an initial understanding of the interactive effects of the combination treatment on this non-*MYCN*-amplified cell line, we evaluated these responses using proliferation and apoptosis assays and surveyed changes in protein expression. Using WST-1 assays, we found that the combination treatment significantly reduced proliferation over lower levels of either single treatment (Fig. 1A, top). The IC_{50} of 0.035 μM for doxorubicin alone decreased to an IC_{50} of 0.014 μM at both concentrations of nutlin-3.

The ELISA apoptosis assay quantifies levels of histone-complexed DNA fragments. Using this assay, we found that while higher doses of the doxorubicin single treatment or the combination treatments both produced increasing levels of apoptosis, the highest dose of the combination treatments induced apoptosis at levels almost 2-fold higher than the highest single dose of doxorubicin (Fig. 1A, bottom). Both single and combination treatments induced the upregulation of p53, Bax and pMdm2. However, only the combination treatment drove the high level expression of the MycN protein (Fig. 1B, top). Moreover, the combination treatment upregulated the expression of the transcriptional factor E2F1 (Fig. 1B, bottom).

Treatment with nutlin-3 and doxorubicin promotes morphological changes in SH-SY5Y cells consistent with apoptosis. SH-SY5Y is a tumorigenic N-type NB cell line, resembling neuroendocrine precursors. We examined changes in cell morphology following a 24 h treatment with single and combination treatments. The doxorubicin treatment alone promoted an increase in cell size (Fig. 2, panel B), while the nutlin-3 treatment induced neuritic-like outgrowths resembling differentiation (Fig. 2, Panel C). The combination treatment induced more pronounced outgrowths, as well as a strongly refractile appearance characteristic of apoptosis (Fig. 2, Panel D).

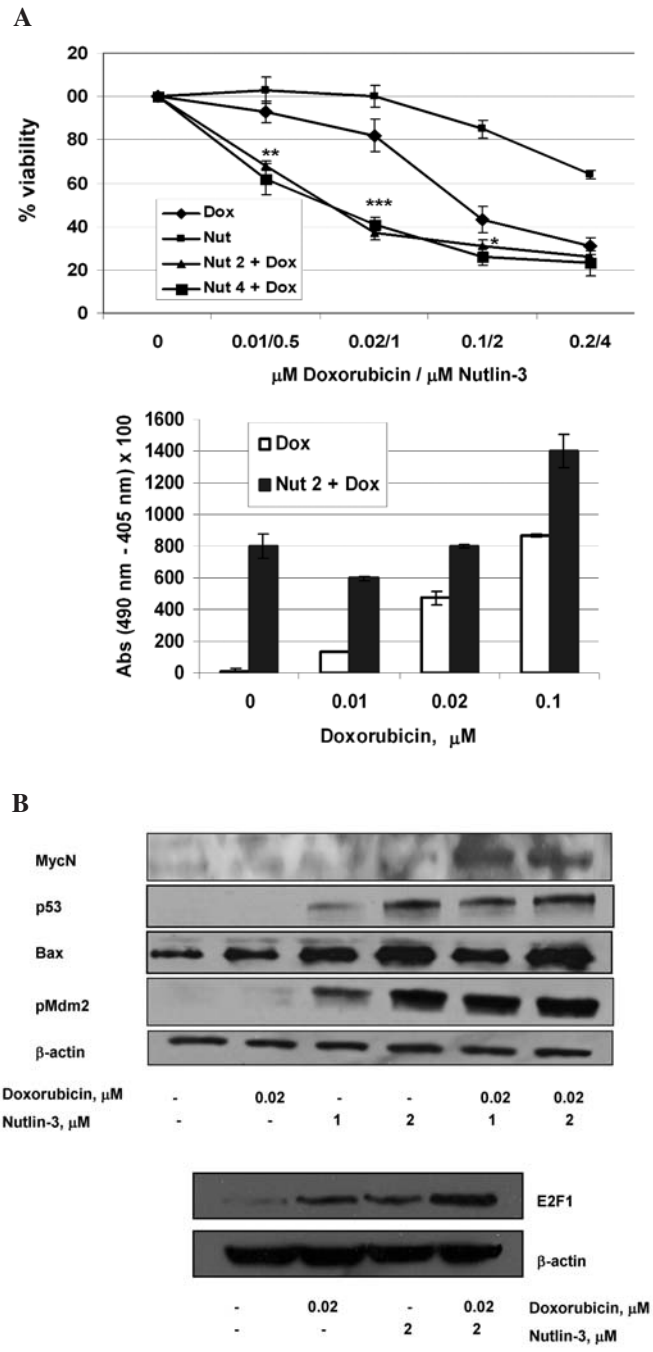

Figure 1. Nutlin-3 and doxorubicin interact to inhibit cell proliferation, induce apoptosis and upregulate the expression of MycN in SH-SY5Y cells. (A) Top panel, dose response for single and combination treatments, as measured by 24 h WST-1 assay, indicating a significant decrease in viability at lower doses of combination treatments vs. single treatments. Bottom panel, ELISA apoptosis assay using quantification of mono- and oligonucleosomes in cytoplasm as measure of apoptosis. Results are representative and show one experiment performed in triplicate. (B) Top panel, 24 h combination treatments of doxorubicin and nutlin-3 induce expression of MycN protein in a non-MycN amplified cell line; single treatments do not. Bottom panel, 24 h combination treatment of doxorubicin and nutlin-3 upregulates expression of E2F1.

The combination treatment markedly induces MycN mRNA, upregulates pro-apoptotic proteins and enhances the expression of favorable neuroblastoma genes, CD44 and EFNB3. To gain insight into the time-dependent nature of gene and protein regulation following the combination treatment, we examined MycN, CD44 and EFNB3 mRNA expression and p53 and Bax protein expression. In response to the combination treatment, MycN mRNA was strongly up-regulated at an early time point, but this expression decreased through 30 h (Fig. 3A, top panel). Simultaneously, p53 protein expression increased markedly by 4 h and remained elevated

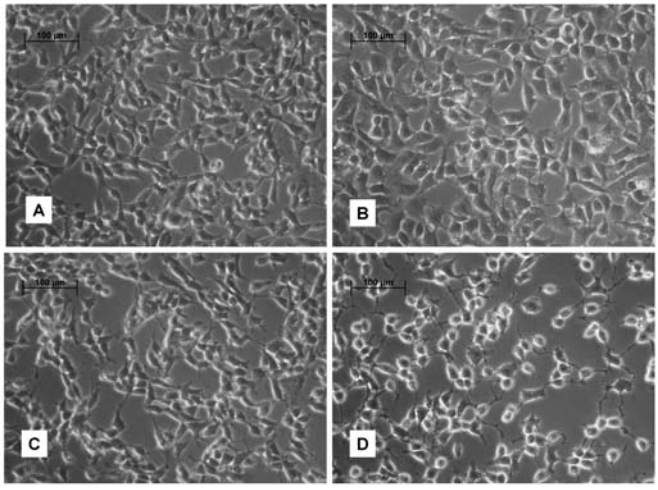

Figure 2. The 24 h doxorubicin/nutlin-3 combination treatment of SH-SY5Y cells promotes alterations in cell morphology consistent with apoptosis. (A) No treatment; (B) treatment with 0.02 μM doxorubicin; (C) treatment with 4 μM nutlin-3; and (D) treatment with 4 μM nutlin-3 + 0.02 μM doxorubicin.

through 30 h (Fig. 3A, bottom panel). In contrast, Bax protein levels rose gradually over this same time period and were highest at 30 h (Fig. 3A, bottom panel).

CD44 expression has been found to inversely correlate with *MYCN* amplification and thus functions as a positive prognostic factor for NB. EFNB3 is a ligand member of the ephrin receptor protein-kinase gene family and is important in neural development; its expression has also been found to correlate with a positive NB outcome. Therefore, we sought to examine the expression of these genes in response to induced MycN expression. CD44 gene expression was highest at 24 h (~10-fold higher than untreated cells) but decreased by 30 h (Fig. 3B, top panel). Conversely, EFNB3 levels were somewhat elevated by 8 h (~1.4-fold higher than untreated cells) and remained at this level through 24 h (Fig. 3B, bottom panel).

Blocking MycN mRNA prevents combination-treatment-induced increases in expression of Bax protein and partially reverses apoptosis. To evaluate the role of increased MycN in the upregulation of Bax protein expression, we used siRNA against *MYCN* to inhibit MycN expression following combination treatment. At 8 h following treatment, MycN levels were substantially reduced from the MycN levels of the negative control RNA transfection (Fig. 4A, top panel); by 24 h, levels of MycN mRNA were comparable for the negative control and MycN-targeted transfections. From Fig. 4A, bottom panel and Fig. 3A (bottom panel), there was an increase in levels of Bax protein between 8-24 h following the combination treatment. Evidence that MycN is responsible for this upregulation was supported by the lack of increased Bax induction following inhibition of MycN mRNA expression (Fig. 4A, bottom).

To confirm that MycN plays a role in driving apoptosis in these combination treatments, we measured levels of cleaved nucleosomes following siRNA-induced MycN silencing, using an ELISA assay. For all three treatments, levels of apoptosis were reduced when MycN RNA expression was inhibited (Fig. 4B).

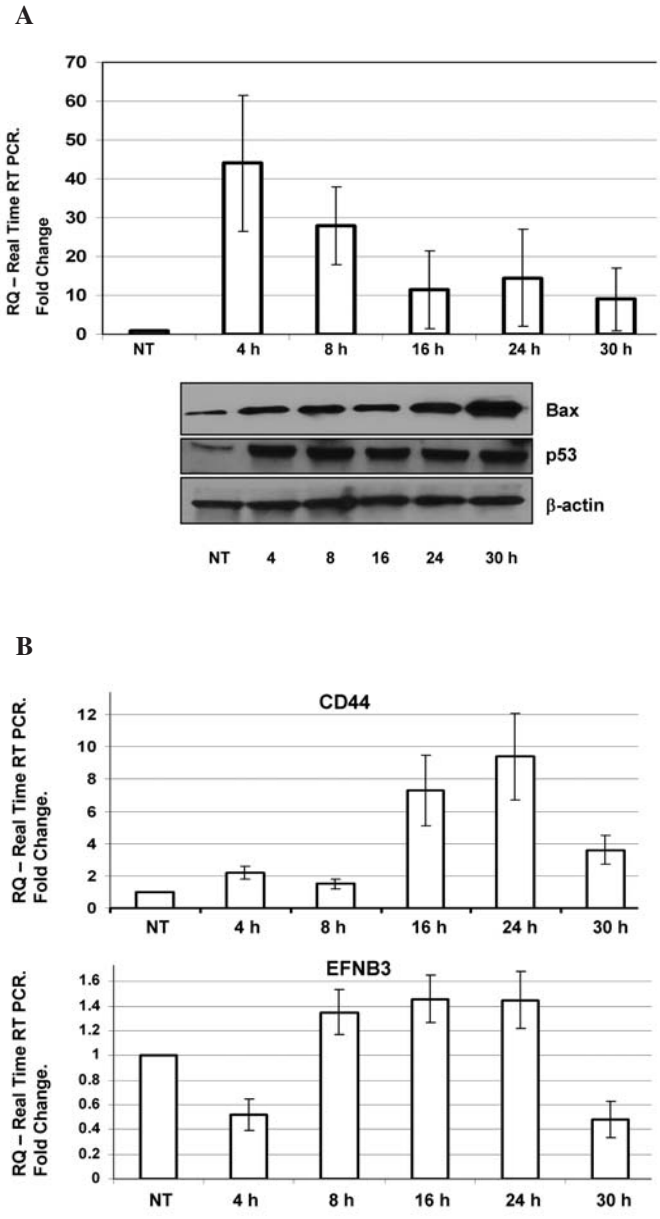

Figure 3. The combination treatment 2 μM nutlin-3 + 0.02 μM doxorubicin strongly induces MycN mRNA, upregulates downstream apoptotic proteins and enhances the mRNA expression of genes favorable to neuroblastoma outcome. (A) Top panel, MycN mRNA expression levels are significantly increased by 4 h following treatment, as measured by real-time RT-PCR. Results are representative and show one experiment run in triplicate. Bottom panel, p53 protein levels are fully upregulated by 4 h, while Bax expression continues to increase through 30 h. (B) Induction of MycN by combination treatment in a non-MycN amplified cell line corresponds to increased mRNA expression of two cell surface markers associated with a positive neuroblastoma prognosis, CD44 and EFNB3. Real-time RT-PCR results are representative and show one experiment performed in triplicate.

In a MycN-inducible cell line (SHEP-Tet21N), MycN interacts with combination treatments to increase levels of pro-apoptotic proteins and enhance the inhibition of proliferation. To evaluate the interactive effects of the combination treatments and the expression of the MycN protein, we used an inducible system of MycN in a non-*MYCN*-amplified cell line (SHEP). MycN protein expression was undetectable in the presence of 10 ng/ml of tetracycline but expressed at high levels in its absence (Fig. 5A, top lane). Levels of p53 were increased in

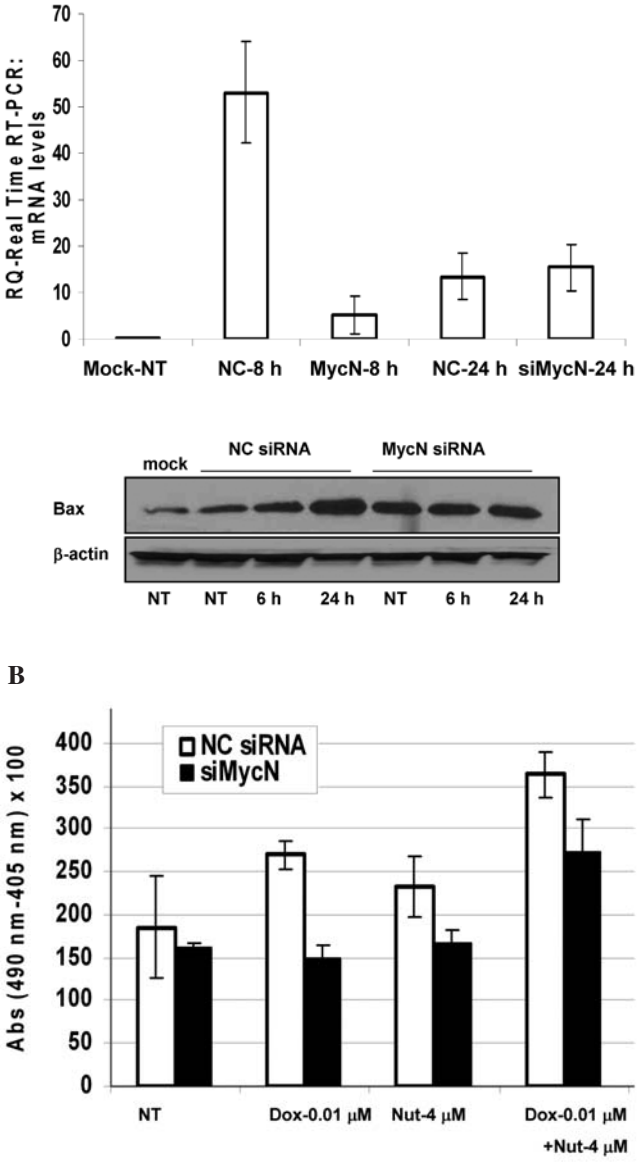

Figure 4. Transfection with MycN siRNA preceeding treatment with 2 μM nutlin-3 + 0.02 μM doxorubicin blocks the increased expression of Bax protein and partially inhibits apoptosis. (A) Top panel, MycN mRNA levels are moderately suppressed by MycN siRNA transfection at 8 h, but return to higher levels by 24 h. A 24 h combination treatment followed an 18 h transfection. NC, negative control siMycN. Real-time RT-PCR results are representative and show one experiment performed in triplicate. Bottom panel, transfection with MycN siRNA in combination-treated cells leads to suppression of Bax upregulation. A 24 h combination treatment followed an 18 h transfection. (B) ELISA apoptosis assay using quantification of mono- and oligonucleosomes in cytoplasm as measure of apoptosis. Apoptosis levels were measured following an 18 h transfection and an additional 24 h combination treatment. Results are representative and show one experiment performed in triplicate.

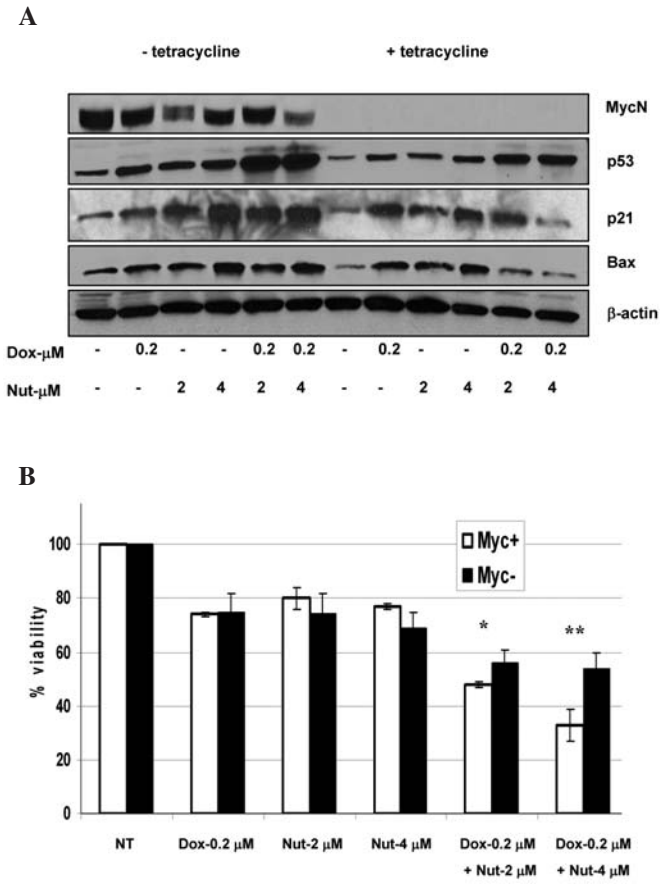

Figure 5. In a MycN-inducible cell line (SHEP-Tet21N), MycN interacts with combination treatments to strongly increase levels of pro-apoptotic proteins and enhance inhibition of proliferation. (A) MycN protein expression upregulates p53, p21 and Bax proteins in response to the nutlin-3/doxorubicin treatments over levels seen without MycN expression. (B) MycN expression in combination with nutlin-3/doxorubicin treatments increases the inhibition of cell proliferation, as measured by 24 h WST-1 assay.

response to the combination treatment in the absence of MycN. However, in the presence of MycN, levels of p53, as well as its two downstream effector targets, p21 and Bax, were dramatically increased in response to combination treatments from levels seen without expression of MycN (Fig. 5A).

To examine a mechanistic role for the MycN protein, we used this cell system to evaluate viability in the presence or absence of MycN with single and combination treatments. Proliferation was significantly reduced in the presence of MycN using 0.2 μM doxorubicin + 2 μM nutlin-3 ($P<0.05$) and 0.2 μM doxorubicin + 4 μM nutlin-3 ($P<0.01$).

Discussion

Previous studies have provided evidence that MycN expression in NB cells without *MYCN* amplification induces apoptosis (23). Others have shown that MycN expression and chemotherapeutics together function to block survival of NB (25). In this study, we have expanded these studies. We provide evidence that the combination treatment of nutlin-3 and doxorubicin is able to upregulate expression of MycN protein, and that high levels of MycN in the absence of *MYCN* amplification interact with this combination to cooperatively induce apoptosis. Thus, this study addresses the upregulation of the pro-apoptotic MycN protein in response to a chemotherapeutic combination treatment, a finding not previously reported.

MycN is a 62-64 kDa nuclear phosphoprotein, with a number of transcription initiation sites controlling basal levels of transcription, including those for the E2F transcription factors which regulate cell cycle progression (8,28). It has long been known that the retinoblastoma (Rb) tumor supppressor/

E2F pathway is an essential link between proliferation and apoptosis and Rogoff *et al* (29) found that E2F1 functions critically in apoptosis, acting, in part, to upregulate expression of Chk2, a checkpoint kinase. Chk2, in turn, is responsible for the phosphorylation and stabilization of E2F1 (30). In addition to the binding of the MycN promoter, E2F1 transactivates pro-apoptotic genes not only by direct interaction (31,32), but also by regulating the splicing of caspases-8 and -9 and BH3 family members (33). E2F1 has long been known to bind to the N-terminal region of Mdm2 and Mdm2 blocks the apoptotic activity of E2F1 by this binding (34). Kitagawa *et al* (34) demonstrated that E2F1 transcriptional activity is essential to nutlin-3-mediated apoptosis. Importantly, E2F1 expression is induced by a large number DNA damaging agents, including doxorubicin (35).

In the current study, we propose that the combined doxorubicin and nutlin-3 treatment induce DNA damage and block Mdm2 binding, leading to E2F1 stabilization and trans-activation of pro-apoptotic genes, including *MYCN*. Here, the binding of p53 and E2F1 to Mdm2 is inhibited by the single nutlin-3 treatment, while doxorubicin treatment alone induces checkpoint kinases by DNA damage, which, in turn, stabilizes free E2F1. The combination treatment promotes high level E2F1 binding to the *MYCN* promoter, enhancing MycN expression and this is borne out by our finding of increased expression of both E2F1 and MycN proteins following this treatment (Fig. 1B). We also demonstrate the upregulation of MycN at the mRNA level (Fig. 3A); this increase in transcription is also likely mediated by the combination treatment-induced high level expression of E2F1.

MycN overexpression in normal, non-oncogenic cells leads to death (36) and Myc is able to bind to the E-boxes of a number of genes for proteins in the apoptosis pathway including p53 and BH3-only proteins (37,38). Increased expression of MycN often promotes caspase-8 activation in neuroblastoma (40). Consistent with this, we noted higher levels of cleaved nucleosomes following the combination treatment (Fig. 1A), an effect that was partially reversed by the knock-down of MycN (Fig. 4B). It is also known that neuroblastomas frequently display mutation of genes directing apoptosis (27). Fulda *et al* have reported that the loss of caspase-8 protein expression occurs in 75% of NB cases, although they find that this loss is not necessarily correlated with *MYCN* amplification (39).

In the current study of NB cell lines lacking *MYCN* amplification, we conjecture that initiator and effector caspase genes are neither mutated nor methylated and that the upregulation of MycN results in high level activation of intact apoptotic cascades. This apoptotic signaling leads to downstream expression of proteins normally seen during NB differentiation including CD44 and EFNB3 (40), by mechanisms that remain elusive; however, one plausible link is that factors that inhibit CD44 and EFNB3 expression in *MYCN*-amplified NB are lacking in these non-*MYCN*-amplified SH-SY5Y cells. The delay in upregulation of CD44 and EFNB3 genes following combination treatment (Fig. 3B) suggests roles as downstream effectors.

Thus, combined doxorubicin and nutlin-3 treatment may provide the basis for a novel therapy strategy for the majority of neuroblastomas lacking *MYCN* amplification, especially as greater insights into the molecular mechanisms involved are gained. We intend to further investigate the efficacy of combination nutlin-3 and doxorubicin for therapy of non-*MYCN*-amplified NB in our preclinical *in vivo* model of this malignancy.

Acknowledgements

We thank Anindya Dasgupta for helpful discussions. This study was supported by CURE Childhood Cancer, Inc.

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